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FOR THE SELECTION OF RECOMBINANT CELLS PRODUCING  
HIGH LEVELS OF A DESIRED GENE PRODUCT  
Docket No. : 1/1411

Commissioner for Patents  
P. O. Box 1450  
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**SUBMISSION OF ENGLISH TRANSLATION**

Sir:

Please find enclosed a copy of an English translation of the German text of the U. S. provisional application filed on July 17, 2003, identifiable by Attorney Docket No. 1/1503 PV, and thereafter accorded Serial No. 60/487,902. Also enclosed is a statement by the translator that the enclosed translation is accurate.

Benefit of this prior provisional is claimed under 35 USC 119(e) and this translation is being submitted pursuant to 37 CFR 1.78(a)(5).

The Commissioner is hereby authorized to charge any other fee which may be required, and to credit any overpayment, to Deposit Account No. 02-2955. A triplicate of this paper is enclosed.

Respectfully submitted,

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## DECLARATION

I, Jane Roberta Mann, B.A., a Translator, of Frank B. Dehn & Co., 59 St Aldates, Oxford OX1 1ST, England, do declare that I have a competent knowledge of the English and German languages and that the document that is annexed hereto is a true and accurate translation of the German text of the U.S. Provisional Application Serial No. 60/487,902 filed July 17, 2003.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

I acknowledge that wilful false statements and the like are punishable by fine or imprisonment, or both [18 U.S.C. 1001] and may jeopardize the validity of the application or any patent issuing therefrom.

A handwritten signature, "Jane Mann", written in cursive script over a solid horizontal line.

Signed this 5th day of November, 2003

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Novel neomycin-phosphotransferase-genes and methods for the selection of  
recombinant cells producing high levels of a desired gene product

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Scope of the Invention

The invention relates to new modified neomycin-phosphotransferase genes and their use in selection methods for high-producing recombinant cells. Accordingly, the present invention also relates to new expression vectors which contain a modified neomycin-phosphotransferase gene, preferably combined with a gene of interest functionally linked to a heterologous promoter. The invention further relates to methods of preparing heterologous gene products using the corresponding high-producing recombinant cells.

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Background to the Invention

Mammalian cells are the preferred host cells for the production of complex biopharmaceutical proteins as the modifications carried out post-translationally are compatible with humans both functionally and pharmacokinetically. The main relevant cell types are hybridoma, myeloma CHO (Chinese Hamster Ovary) cells and BHK (Baby Hamster Kidney) cells. The cultivation of the host cells is increasingly carried out under serum- and protein-free production conditions. The reasons for these are the concomitant cost reduction, the reduced interference in the purification of the recombinant protein and the reduction in the potential for the introduction of pathogens (e.g. prions and viruses). The use of CHO cells as host cells is becoming more widespread as these cells adapt to suspension growth in serum- and protein-free medium and are also regarded and accepted as safe production cells by the regulatory authorities.

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In order to produce a stable mammalian cell line which expresses a heterologous gene of interest (GOI), the heterologous gene is generally inserted in the desired cell line together with a selectable marker gene such as e.g. neomycin

phosphotransferase (NPT) by transfection. The heterologous gene and the selectable marker gene can be expressed in a host cell starting from one individual or separate cotransfected vectors. Two to three days after transfection the transfected cells are transferred into medium containing a selective agent, e.g. G418 when using  
5 neomycin phosphotransferase-gene (NPT gene), and cultivated for some weeks under these selective conditions. The emerging resistance cells which have integrated the exogenous DNA can be isolated and investigated for expression of the desired gene product (of the GOI).

10 A major problem in establishing cell lines with a high expression of the desired proteins arises from the random and undirected integration of the recombinant vector into transcriptionally -active or -inactive loci in the host cell genome. As a result a population of cells is obtained which have completely different expression rates of the heterologous gene, the productivity of the cells generally following normal  
15 distribution. In order to identify cell clones which have a very high expression of the heterologous gene of interest it is therefore necessary to examine and test a large number of clones, which is time consuming, labour intensive and expensive. Improvements to the vector system used for transfection therefore set out to increase the proportion of high producers in the transfected cell population by suitable  
20 selection strategies and thereby reduce the expenditure and work involved in clone identification. The development of such an expression system is the subject of the present invention.

The amino glycoside-3'-phosphotransferase II enzyme (neomycin-  
25 phosphotransferase) (EC27195) the gene of which is transposon 5- associated in *Escherichia coli* is used as a selectable marker in a number of organisms (e.g. bacteria, yeasts, plants and mammalian cells). This enzyme confers resistance to various aminoglycoside antibiotics such as neomycin, kanamycin and G418, by inactivating the antibiotics by transferring the terminal phosphate from ATP to the 3'  
30 hydroxyl group of the aminohexose ring I. In addition to the wild-type neomycin phosphotransferase some mutants are known which have reduced phosphotransferase activity and hence reduced resistance to aminoglycoside

antibiotics in bacteria (Blázquez et al., 1991; Kocabiyik et al., 1992; Yenofsky et al., 1990) and in slices of leaf from tobacco (Yenofsky et al., 1990).

One of these mutants (Glu182Asp) was used as a marker for selecting embryonic stem cells, the neomycin phosphotransferase gene being integrated into the c-myc gene by targeted homologous recombination (gene targeting) (Hanson et al., 1995). The authors restrict themselves to the use of the modified enzyme for gene targeting.

Patent application WO 99/53046 describes the expression of a modified neomycin phosphotransferase gene (Asp261Asn) in production-relevant mammalian cells. The authors describe a non-cloning method for expression of a gene of interest in mammalian cells. By cotransfection of the cells with three individual DNA fragments which code for a promoter element, a gene of interest and a selectable marker coupled with an IRES ("Internal ribosomal entry site") element, it is possible to deliberately grow cells, under selection pressure, in which all three DNA fragments are combined as a functional bicistronic transcription unit (promoter gene of interest-IRES-neomycin-phosphotransferase gene). The arrangement of the elements only occurs in the transfected cell, so that only a few cells show the correct arrangement of the elements. Moreover, after gene amplification, using an amplifiable selectable marker, no high producing clones can be generated. After repeated selection and gene amplification the cells generated exhibited at most 6 pg of protein per cell per day (6pg/cell/day).

None of the publications discloses modified neomycin phosphotransferase genes with particular suitability for the preparation of a high expression vector system for mammalian cells which makes it possible to develop high producing cells in order to prepare recombinant biopharmaceutical proteins which contain one or more complete functional transcription units both for one or more genes of interest and also for a modified neomycin phosphotransferase gene with reduced antibiotic resistance. The DNA construct described in WO 99/53046 contains only a promoter-less neomycin gene functionally linked to the gene for dihydrofolate reductase (DHFR).

There is therefore a need to make suitable modified neomycin phosphotransferase genes available, particularly for the development of corresponding high expression vector systems for biopharmaceutical processes. The problem of the present invention was therefore to provide corresponding new modified neomycin

5 phosphotransferase genes, expression vectors which contain a modified neomycin phosphotransferase gene and a gene of interest functionally linked to a heterologous promoter, a method of selection for high producing recombinant cells, preferably for mammalian cells, and a process for producing heterologous gene products.

10 Surprisingly, within the scope of the present invention, it has been possible to produce and identify new modified highly selective neomycin phosphotransferase genes which are characterised by their particular suitability for the selection of high producing cells.

15 Summary of the Invention

The present invention provides new modified neomycin phosphotransferase genes. Surprisingly, it has been found that an enrichment of transfected mammalian cells with high expression rates of the co-integrated gene of interest could be achieved by

20 using the modified neomycin phosphotransferase genes described hereinafter as selectable markers. Compared with the use of the wild-type neomycin phosphotransferase as selectable marker, after transfection with one of the new neomycin phosphotransferase genes according to the invention the cells exhibited a productivity of a protein (an antibody) which was increased by a factor 1.4 to 14.6.

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The modified neomycin phosphotransferase genes according to the invention are preferably mutants which code for a different amino acid from the wild-type gene at amino acid position 91, 182, 198, 227, 240 or 261. In a preferred embodiment the neomycin phosphotransferase gene according to the invention is the mutant

30 Glu182Gly, Trp91Ala, Val198Gly, Asp227Val, Asp227Gly, Asp261Asn, Asp261Gly or Phe240Ile. For selecting high producing mammalian cells it has proved particularly suitable to use the mutants Trp91Ala, Asp227Val, Asp261Asn, Asp261Gly and

Phe240Ile, while the mutants Asp227Val and Asp261Gly in turn gave cell clones with the highest productivity.

The high-producing cells were obtained by the use of a eukaryotic expression vector which contains a heterologous gene of interest functionally linked to a heterologous promoter and a modified neomycin phosphotransferase gene according to the invention. The expression vector preferably contains other regulatory elements, e.g. one or more enhancers functionally linked to the promoter or promoters. Expression vectors are also preferred which additionally contain a gene for a fluorescent protein which is functionally linked to the gene of interest and the heterologous promoter, preferably via an internal ribosomal entry site (IRES), which enables bicistronic expression of the gene which codes for a fluorescent protein and of the gene which codes for a protein/product of interest, under the control of the heterologous promoter. Particularly suitable are expression vectors in which the heterologous gene of interest is under the control of the ubiquitin/S27a promoter.

The invention also relates to expression vectors which instead of the gene of interest contain a multiple cloning site for incorporating such a gene, i.e. a sequence section with multiple recognition sequences for restriction endonucleases.

In another aspect the invention relates to recombinant mammalian cells which contain one of the abovementioned modified neomycin phosphotransferase genes according to the invention. In addition the present invention relates to recombinant mammalian cells which have been transfected with one of the expression vectors according to the invention. These are preferably recombinant rodent cells, of which recombinant hamster cells such as e.g. CHO cells or BHK cells are particularly preferred. In another preferred embodiment the said recombinant cells are additionally transfected with the gene for an amplifiable selectable marker, e.g. with the gene of dihydrofolate reductase (DHFR).

The invention also relates to a process for enriching recombinant mammalian cells which express a modified neomycin phosphotransferase gene, characterised in that (i) a pool of mammalian cells is transfected with a gene for a modified neomycin

phosphotransferase, which has only 1 to 80%, preferably only 1 to 60%, more preferably only 1.5 to 30%, most preferably only 1.5 to 26% of the activity and/or one of the modifications described above; (ii) the mammalian cells are cultivated under conditions which allow expression of the modified neomycin phosphotransferase gene; and (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the neomycin phosphotransferase gene.

The invention also relates to a process for the expression of at least one gene of interest in recombinant mammalian cells, characterised in that (i) a pool of mammalian cells is transfected with at least one gene of interest and one gene for a modified neomycin phosphotransferase which exhibits only 1 to 80%, preferably only 1 to 60%, more preferably only 1.5 to 30%, most preferably only 1.5 to 26% of the activity and/or one of the modifications described above; (ii) the cells are cultivated under conditions which allow expression of the gene or genes of interest and the modified neomycin phosphotransferase gene; (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells and gives preference to the growth of those cells which express the neomycin phosphotransferase gene; and (iv) the protein or proteins of interest is or are obtained from the mammalian cells or from the culture supernatant.

The present invention further relates to a process for obtaining and selecting recombinant mammalian cells which express at least one heterologous gene of interest, which is characterised in that (i) recombinant mammalian cells are transfected with an expression vector according to the invention which in addition to the gene of interest and the modified neomycin phosphotransferase gene codes for a fluorescent protein; (ii) the mammalian cells are cultivated under conditions which allow expression of the gene or genes of interest, the gene which codes for a fluorescent protein and the modified neomycin phosphotransferase gene; (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells and gives preference to the growth



of those cells which express the neomycin phosphotransferase gene; and (iv) the mammalian cells are sorted by flow-cytometric analysis.

If the mammalian cells have additionally been transfected with a gene for an amplifiable selectable marker gene, e.g. the DHFR gene, it is possible to cultivate the mammalian cells under conditions in which the amplifiable selectable marker gene is also expressed, and to add to the culture medium a selecting agent which results in amplification of the amplifiable selectable marker gene.

Preferably, the processes according to the invention are carried out with mammalian cells which are adapted to growth in suspension, i.e. with mammalian cells which are cultivated in a suspension culture. Other embodiments relate to processes in which the mammalian cells, preferably those which are adapted to growth in suspension, are cultivated under serum-free conditions.

#### Description of the Figures

**Figure 1** shows a diagrammatic representation of the base vectors used to express the recombinant proteins in CHO-DG44 cells. "P/E" is a combination of CMV enhancer and hamster-ubiquitin/S27a promoter, "P" on its own indicates a promoter element and "T" is a termination signal for transcription, which is needed for the polyadenylation of the transcribed mRNA. The position and direction of transcription initiation within each transcription unit is indicated by an arrow. For cloning the heterologous genes a sequence region with multiple cutting sites for restriction endonucleases (multiple cloning sites - MCS) is inserted after the promoter element. The amplifiable selectable marker dihydrofolate reductase is abbreviated to "dhfr" and the selectable marker neomycin phosphotransferase is abbreviated to "npt" (npt wild-type or npt mutant). The "IRES" element coming from the encephalomyocarditic virus acts as an internal ribosomal entry site within the bicistronic transcription unit and enables translation of the following green fluorescent protein "GFP".

**Figure 2** shows a diagrammatic view of the eukaryotic expression vectors which code for a subunit of a monoclonal antibody and are used to transfect CHO-DG44

cells. "P/E" is a combination of CMV enhancer and hamster ubiquitin/S27a promoter, "P" on its own is a promoter element and "T" is a termination signal for the transcription which is needed for the polyadenylation of the transcribed mRNA. The position and direction of transcription initiation within each transcription unit is indicated by an arrow. The amplifiable selectable marker dihydrofolate reductase is abbreviated to "dhfr" and the selectable marker neomycin phosphotransferase is abbreviated to "npt". The NPT mutants E182G (SEQ ID NO:3), W91A (SEQ ID NO:5), V198G (SEQ ID NO:7), D227A (SEQ ID NO:9), D227V (SEQ ID NO:11), D261G (SEQ ID NO:13), D261N (SEQ ID NO:15) and F240I (SEQ ID NO:17) contain a point mutation which results in a modified amino acid (given in 1-letter code) at the position indicated. The IRES element originating from the encephalomyocarditis virus acts as an internal ribosomal entry site within the bicistronic transcription unit and permits translation of the following green fluorescent protein "GFP", whereas "HC" and "LC" code for the heavy and light chains, respectively, of a humanised monoclonal IgG2 antibody.

**Figure 3** shows the part of the sequence of the neomycin phosphotransferase (npt) gene in which the point mutations have been inserted by PCR with mutagenic primers. The capital letters indicate the nucleotide sequence of the npt coding region whereas the small letters indicate the flanking non-coding nucleotide sequences. The amino acid sequence predicted from the nucleotide sequence (3-letter code) is shown above the coding nucleotide sequence. Arrows indicate the direction, length and position of the primers used, the arrows with solid lines indicating the mutagenic forward primers, the broken lines indicating the mutagenic reverse primers, the dotted lines indicating the primer Neofor5 (SEQ ID NO:19) or Neofor2 (SEQ ID NO:21) located upstream of the npt gene or the mutation site, respectively, and the dot-dash line indicating the primer Neorev5 (SEQ ID NO:20) or IC49 (SEQ ID NO:22) located downstream of the npt gene or the mutation site, respectively. The nucleotides exchanged with respect to the wild-type sequence are emphasised above and below the arrows.

**Figure 4** shows conserved domains and the position of the inserted NPT mutations within the NPT amino acid sequence. On the basis of sequence homologies

between different aminoglycoside-modified enzymes, different conserved domains were identified within the NPT protein sequence (shown in grey) The three motifs in the C-terminal region of the enzyme obviously have special functions. Motifs 1 and 2 are presumably involved in the catalytic transfer of the terminal phosphate in the ATP catalysis or the nucleotide binding, whereas motif 3 is thought to have a function in the ATP hydrolysis and/or the change in conformation in the enzyme-aminoglycoside complex. Amino acids which occur in at least 70% of the aminoglycoside-modifying enzymes are emphasised in bold type. The singly underlined amino acids are assigned to the same group on the basis of their similarity and occur in at least 70% of the aminoglycoside-modifying enzymes. Amino acids marked with an asterisk indicate the position of the mutation sites.

In **Figure 5** the influence of the NPT mutations on the selection of stably transfected mAb expressing cells was investigated. For this, CHO-DG44 cells were transfected with the plasmid combinations pBIDG-HC/pBIN-LC (NPT-wild-type), pBIDG-HC/pBIN1-LC (NPT mutant Asp182Gly), pBIDG-HC/pBIN2-LC (NPT mutant Trp91Ala), pBIDG-HC/pBIN3-LC (NPT mutant Val198G), pBIDG-HC/pBIN4-LC (NPT mutant Asp227Ala), pBIDG-HC/pBIN5-LC (NPT mutant Asp227Val), pBIDG-HC/pBIN6-LC (NPT mutant Asp261Gly), pBIDG-HC/pBIN7-LC (NPT mutant Asp261Asn) or pBIDG-HC/pBIN8-LC (NPT mutant Phe240Ile), which differ from one another only in the NPT gene (wild-type or mutant) used as a selectable marker. The concentration in the cell culture supernatant of the recombinant monoclonal IgG2 antibody produced was determined by ELISA and the specific productivity per cell and per day was calculated. In all, 5 pools were set up for each vector combination. The bars represent the averages of the specific productivity or of the titre of all the pools in the Test from 6 cultivation runs in 75cm<sup>2</sup> flasks. To calculate the relative titres or the relative specific productivities the averages of the pools selected with the NPT wild-type gene were taken as 1.

**Figure 6** shows the enrichment of cells with a higher GFP expression in transfected cell pools by using the NPT mutants according to the invention as selectable markers. For this, CHO-DG44 cells were transfected with the plasmid combinations pBIDG-HC/pBIN-LC (NPT-wild-type), pBIDG-HC/pBIN1-LC (NPT mutant Asp182Gly),

pBIDG-HC/pBIN2-LC (NPT mutant Trp91Ala), pBIDG-HC/pBIN3-LC (NPT mutant Val198G), pBIDG-HC/pBIN4-LC (NPT mutant Asp227Ala), pBIDG-HC/pBIN5-LC (NPT mutant Asp227Val), pBIDG-HC/pBIN6-LC (NPT mutant Asp261Gly), pBIDG-HC/pBIN7-LC (NPT mutant Asp261Asn) or pBIDG-HC/pBIN8-LC (NPT mutant Phe240Ile) (5 pools in each case), which differ from one another only in the NPT gene (wild-type or mutant) used as the selectable marker. Moreover, the pBIDG vectors also contained the GFP as marker gene. After 2 to 3 weeks' selection of the transfected cell pools in HT-free medium with the addition of G418, the GFP fluorescence was measured by FACS analysis. Every graph, with the exception of the non-transfected CHO-DG44 cells used as a negative control, represents the average GFP fluorescence from the pools which had been transfected with the same plasmid combination.

**Figure 7** shows in tabulated form the enzyme activity of the NPT mutants according to the invention compared with the NPT wild-type, determined in a dot assay. For this, cell extracts were prepared from two different cell pools (pool 1 and 2) expressing mAb, which had been transfected and selected either with the NPT wild-type gene (SEQ ID NO:1) or with the NPT mutants E182G (SEQ ID NO:3), W91A (SEQ ID NO:5), V198G (SEQ ID NO:7), D227A (SEQ ID NO:9), D227V (SEQ ID NO:11), D261G (SEQ ID NO:13), D261N (SEQ ID NO:15) and F240I (SEQ ID NO:17)Glu182Asp or Asp227Gly. Non-transfected CHO-DG44 cells were used as negative control. G418 was used as the substrate in the phosphorylation assay. The extracts were filtered through a sandwich of P81 phosphocellulose and nitrocellulose membrane in a 96 well vacuum manifold. Proteins phosphorylated by protein kinases and also non-phosphorylated proteins bind to the nitrocellulose, whereas phosphorylated and non-phosphorylated G418 passes through the nitrocellulose and binds to the phosphocellulose. The radioactive signals were detected and quantified using a phosphoimager. The signals which had been obtained with 5 µg of extract were used to calculate the percentage enzyme activity. The percentage enzyme activities denote the average of the NPT mutants from 2 cell pools expressing mAb, the enzyme activity of wild-type NPT being taken as 100%.

**Figure 8** shows the Northern Blot analysis of NPT expression and the number of NPT gene copies in the transfected cell pools. For this, total RNA was prepared from two different cell pools expressing mAb (pools 1 and 2) which were transfected and selected either with the NPT wild-type gene (SEQ ID NO:1) or with the NPT mutants  
5 E182G (SEQ ID NO:3), W91A (SEQ ID NO:5), V198G (SEQ ID NO:7), D227A (SEQ ID NO:9), D227V (SEQ ID NO:11), D261G (SEQ ID NO:13), D261N (SEQ ID NO:15) and F240I (SEQ ID NO:17). Untransfected CHO-DG44 cells were used as the negative control. 30 µg of RNA was hybridised with a FITC-dUTP-labelled PCR product which comprised the coding region of the NPT gene. The exposure time of  
10 the X-ray film was 1 hour. In all the transfected cells a specific singular NPT transcript of about 1.3 kb was detected. In order to determine the npt gene copy number, in a dot blot analysis, genomic DNA was isolated from the abovementioned cell pools (pool 1 and 2) expressing mAb.

10 µg, 5 µg, 2.5 µg, 1.25 µg, 0.63 µg and 0.32 µg of genomic DNA was hybridised  
15 with an FITC-dUTP-labelled PCRproduct which included the coding region of the NPT gene. Untransfected CHO-DG44 cells were used as the negative control. The plasmid pBIN-LC was used as the standard (320 pg, 160 pg, 80 pg, 40 pg, 20 pg, 10 pg, 5 pg, 2.5 pg). The copy number of the npt genes in the cell pools was calculated using the standard series which had been determined from the signal intensities  
20 measured for the titrated plasmid-DNA.

#### Detailed Description of the Invention and Preferred Embodiments

The following information on the amino acid positions relates in each case to the  
25 position of the amino acid as coded by the wild-type neomycin phosphotrasferase gene with SEQ ID NO:1. By a "modified neomycin phosphotransferase gene" is meant a nucleic acid which codes for a polypeptide with neomycin phosphotransferase activity, the polypeptide having a different amino acid from the wild-type protein at at least one of the amino acid positions described more fully in  
30 the specification which are homologous to the wild-type protein with SEQ ID NO:2. In this context the term "homologous" means that the sequence region carrying the mutation can be brought into correspondence with a reference sequence, in this case the sequence of the wild-type neomycin phosphotransferase according to SEQ ID

NO:2, using so-called standard "alignment" algorithms, such as for example "BLAST" (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410; Gish, W. & States, D.J. (1993) "Identification of protein coding regions by database similarity search." Nature Genet. 3:266-272; Madden, T.L., Tatusov, R.L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131-141; Zhang, J. & Madden, T.L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." Genome Res. 7:649-656; Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402). Sequences are in correspondence when they correspond in their sequence order and can be identified using the standard "alignment" algorithms.

The present invention provides new modified neomycin phosphotransferase genes and methods of preparing and selecting mammalian cell lines which allow a high expression of heterologous gene products, preferably biopharmaceutically relevant polypeptides or proteins. The processes according to the invention are based primarily on the selection of cells which in addition to the gene of interest express a neomycin phosphotransferase gene according to the invention which gives the transfected cells a selective advantage over non-transfected cells. Surprisingly, it has been found that the use of the modified neomycin phosphotransferase genes (mNPT genes) according to the invention described herein has a substantial selective advantage over the wild-type neomycin phosphotransferase gene (wtNPT gene). This particularly relates to the use of mutants which have a lower enzyme activity compared with wtNPT.

#### Neomycin Phosphotransferase genes modified according to the Invention

It has proved particularly suitable to use modified NPT genes which code for an NPT having only 1 to 80%, preferably only 1 to 60% of the enzyme activity of wtNPT. Preferred NPT mutants are those which have only 1 to 30% of the enzyme activity of wtNPT, while those which have only 1.5 to 26% of the enzyme activity of wtNPT are

particularly preferred. The enzyme activity of an NPT can be determined for example in a dot assay as described in Example 3 and given as Method 5.

The term wild-type neomycin phosphotransferase refers to a neomycin phosphotransferase gene which codes for the aminoglycoside-3'-phosphotransferase II enzyme (EC 2.7.1.95) the gene of which is naturally transposon 5-associated in *Escherichia coli*, and contains for example the amino acid sequence given in SEQ ID NO:2 or is coded by the nucleotide sequence given in SEQ ID NO:1. This enzyme gives resistance to various aminoglycoside antibiotics such as neomycin, kanamycin and G418, by inactivating the antibiotics by the transfer of the terminal phosphate of ATP to the 3' hydroxyl group of the amino hexose ring I. The term wtNPT also refers to all NPTs which have a comparable enzyme activity to the NPT coded by SEQ ID NO:1. This includes in particular those NPTs in which the enzymatically active centre which catalyses the transfer of a terminal phosphate from ATP to a substrate is present in an identical or nearly identical conformation (Shaw et al., 1993; Hon et al; 1997; Burk et al., 2001) and thus has a comparable enzyme activity to an enzyme which contains the amino acid sequence of SEQ ID NO:2. A wtNPT has a comparable enzyme activity if it exhibits about 81 to 150%, preferably 90 to 120% of the enzyme activity displayed by an NPT defined by SEQ ID NO:2, the activity being determined in the dot assay described in Example 3 and referred to as Method 5.

Fundamentally preferred are mutants wherein the reduction in the enzyme activity compared with wtNPT is based on a modification of the amino acid sequence, e.g. on the substitution, insertion or deletion of at least one or more amino acids. Deletion, insertion and substitution mutants can be produced by "site-specific mutagenesis" and/or "PCR-based mutagenesis techniques". Suitable methods are described for example by Lottspeich and Zorbas (1998)(Chapter 36.1 with other references).

Surprisingly, it has been found that if neomycin phosphotransferase mutants are used as selectable markers in which at least the amino acid tryptophan at amino acid position 91, the amino acid glutamic acid at amino acid position 182, the amino acid valine at amino acid position 198, the amino acid aspartic acid at amino acid position 227, the amino acid aspartic acid at amino acid position 261 or the amino acid

phenylalanine at amino acid position 240 has been altered compared with wtNPT, it is possible to achieve particularly effective enrichment of transfected mammalian cells with a high expression rate for the co-integrated gene of interest. Accordingly, mutants which affect the amino acids at positions 91, 182, 198, 227 and/or 240 are preferred. Particularly advantageous are substitution mutants, i.e. mutants in which the amino acids occurring at this location in the wild-type have been replaced by another amino acid. Even more preferred are corresponding substitution mutants in which a change in the corresponding amino acid leads to a reduction in the enzyme activity compared with wt-NPT to 1 to 80%, preferably to 1 to 60%, more preferably to 1.5 to 30%, most preferably to 1.5% to 26%. Particularly preferred are modified NPT genes in which the amino acid 91, 227, 261 and/or 240 has been modified accordingly so that the enzyme activity compared with the wt-NPT is only 1 to 80%, preferably only 1 to 60%, more preferably only 1.5 to 30%, most preferably only 1.5% to 26%. Most preferred is a substitution mutant in which the amino acid at amino acid position 227 has been modified in the form such that the enzyme activity of the modified NPT is less than 26%, preferably between 1 and 20%, more preferably between 1 and 16% compared with the wt-NPT.

According to another embodiment of the present invention, advantageous mutants are those which, by comparison with wtNPT, code for glycine, alanine, valine, leucine, isoleucine, phenylalanine or tyrosine at amino acid positions 91, 182 or 227. Also preferred are modified NPT genes which, by comparison with wtNPT, code for glycine, alanine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, asparagine, glutamine or aspartic acid at amino acid position 261. In particular, it has been found that with the mutants Glu182G, Trp91Ala, Val198Gly, Asp227Ala, Asp227Val, Asp261Gly, Asp261Asn and Phe240Ile as selectable markers it was possible to achieve an enrichment of transfected mammalian cells with high expression rates of the co-integrated gene of interest, with the result that these mutants are particularly preferred. Still more preferred are the mutants Asp227Val, Asp261Gly, Asp261Asn, Phe240Ile and Trp91Ala, as the best enrichment rates are achieved using them.

The amino acids at positions 182 and 227 based on the wild-type are non-conserved amino acids which are located outside the three conserved motifs in the C-terminal



region of the aminoglycoside-3'-phosphotransferases. The amino acid at position 91 also belongs to the non-conserved amino acids and is located outside one of the conserved motifs in the N-terminal region of the aminoglycoside-3'-phosphotransferases. By contrast the amino acids at positions 198 and 240 are conserved amino acids in the C-terminal region of the NPT, but are nevertheless outside the conserved motifs. By contrast, the amino acid at position 261 is a conserved amino acid in the third conserved motif of the C-terminal region (Shaw et al., 1993; Hon et al., 1997; Burk et al., 2001).

Compared with the use of wtNPT as selectable marker the cells in the case of the Glu182Gly and Val198Gly mutant showed a productivity increased by a factor of 1.4, in the case of the Asp227Ala or Trp91Ala mutant productivity was increased by a factor of 2.2 or 4, in the case of the Phe240Ile or Asp261Asn mutant productivity was increased by a factor of 5.7 or 7.3 and in the case of the Asp261Gly or Asp227Val mutant it was even increased by a factor of 9.3 or 14.6. To express the multi-chained protein, an antibody, co-transfection was carried out. The two protein chains were each expressed by their own vector, one vector additionally coding for the NPT gene while the other vector coded for the amplifiable selectable dihydrofolate reductase gene.

The present invention thus relates to a process for enriching for recombinant mammalian cells which express a modified neomycin phosphotransferase gene, characterised in that (i) a pool of mammalian cells is transfected with a gene for a modified neomycin phosphotransferase which has only 1 to 80%, preferably 1 to 60%, more preferably 1.5 to 30%, most preferably 1.5 to 26% of the activity of wild-type neomycin phosphotransferase and/or one of the modifications described herein; (ii) the mammalian cells are cultivated under conditions which allow expression of the modified neomycin phosphotransferase gene; and (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the neomycin phosphotransferase gene.

Particularly preferred is a corresponding process which uses a modified NPT gene described in more detail in this application, particularly if the modified NPT gene used codes for a modified NPT which, by comparison with the wild-type gene, codes for alanine at amino acid position 91, for glycine at amino acid position 182 or 198, for alanine or valine at amino acid position 227, for glycine or asparagine at amino acid position 261 or for isoleucine at amino acid position 240. Still more preferred are NPT genes which, by comparison with the wild-type gene, code for valine at amino acid position 227 and /or for glycine and/or asparagine at amino acid position 261.

The present invention further relates to eukaryotic expression vectors which contain (i) a heterologous gene of interest functionally linked to a heterologous promoter and (ii) a modified neomycin phosphotransferase gene according to the invention which codes for a neomycin phosphotransferase which has low enzyme activity compared with wild-type neomycin phosphotransferase. By a "low" or "lower" enzyme activity for the purposes of the invention is meant an enzyme activity which corresponds to at most 80%, preferably 1 to 80%, more preferably only 1 to 60% of the enzyme activity of wtNPT. According to one embodiment of the present invention "lower enzyme activity" denotes an enzyme activity of 1 to 30%, preferably 1.5 to 26% compared with wild-type neomycin phosphotransferase.

A preferred expression vector contains a modified NPT gene which codes for a modified NPT which has only 1 to 80%, preferably only 1 to 60% of the enzyme activity of wtNPT. Also preferred are expression vectors with modified NPT genes which code for mutants having only 1 to 30% of the enzyme activity of wtNPT.

Particularly preferred are those expression vectors which contain a modified NPT gene which code for mutants having only 1.5 to 26% of the enzyme activity of wtNPT, the activity being determined in the dot assay described in Example 3 and referred to as method 5.

In another embodiment of the invention the expression vectors contain genes of modified NPT which have been modified, compared with wtNPT, at amino acid position Trp91, Glu182, Val198, Asp227, Phe240 or at position Asp261. In this context, NPT mutants are preferred which are modified at position Trp91, Glu182,

Val198, Asp227, Phe240 or Asp261 and have only 1 to 80%, preferably only 1 to 60%, more preferably only 1.5 to 30%, and most preferably only 1.5 to 26% of the enzyme activity of wtNPT. Preferably the amino acids Trp91, Glu182 or Asp227 may each be replaced by glycine, alanine, valine, leucine, isoleucine, phenylalanine or tyrosine at the corresponding position. Preferably the glutamic acid at position 182 may also be replaced by aspartic acid, asparagine, glutamine or another preferably negatively charged amino acid. Also preferred are modified NPT genes which code for glycine, alanine, leucine, isoleucine, phenylalanine, tyrosine or tryptophan at amino acid position 198 compared with wtNPT. In addition, modified NPT genes are preferred which code for glycine, alanine, valine, isoleucine, tyrosine or tryptophan at amino acid position 240 compared with wtNPT. Also preferred are modified NPT genes which code for glycine, alanine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, asparagine, glutamine or aspartic acid at amino acid 261 compared with wtNPT. It is particularly preferred to use a mutant wherein the aspartic acid at position 227 is replaced by glycine, alanine, valine, leucine or isoleucine, the aspartic acid at position 261 is replaced by an alanine, valine, leucine, isoleucine or glutamine, particularly by glycine or asparagine.

Particularly preferred are expression vectors which contain modified NPT genes which code for a Glu182Gly, Trp91Ala, Val198Gly, Asp227Ala, Asp227Val, Asp261Gly, Asp261Asn or Phe240Ile mutant, which in the case of the Glu182Gly mutant contains the amino acid sequence of SEQ ID NO:4, in the case of the Trp91Ala mutant contains the amino acid sequence of SEQ ID NO:6, in the case of the Val198Gly mutant contains the amino acid sequence of SEQ ID NO:8, in the case of the Asp227Ala mutant contains the amino acid sequence of SEQ ID NO:10, in the case of the Asp227Val mutant contains the amino acid sequence of SEQ ID NO:12, in the case of the Asp261Gly mutant contains the amino acid sequence of SEQ ID NO:14, in the case of the Asp261Asn mutant contains the amino acid sequence of SEQ ID NO:16 and in the case of the Phe240Ile mutant contains the amino acid sequence of SEQ ID NO:18. Most preferred is an expression vector using an Asp227Val, Asp261Gly, Asp261Asn, Phe240Ile or Trp91Ala mutant, particularly if it contains the amino acid sequence given in SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18 or SEQ ID NO:6 or also if it is coded by the nucleic acid

sequence given in SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 or SEQ ID NO:5 or contains it.

In addition the present invention provides for the first time modified neomycin  
5 phosphotransferase genes and the gene products thereof which compared with  
wtNPT code for a different amino acid than the wt amino acid at amino acid position  
Trp91, Val198 or Phe240. The present invention particularly provides for the first time  
Trp91, Val198 or Phe240 mutants which have a reduced enzyme activity compared  
10 with wtNPT. The modified NPTs described here and made available within the scope  
of the invention preferably code for alanine at amino acid position 91, for glycine at  
position 198 and for isoleucine at position 240. Furthermore, the present invention  
provides for the first time NPT mutants which, compared with wtNPT, code for glycine  
at position 182, for alanine or valine at position 227 and for glycine at position 261.  
Both the genes and the gene products (enzymes) are provided for the first time within  
15 the scope of the invention. In this context the present invention provides for the first  
time modified NPT with the amino acid sequences according to SEQ ID NO:4, SEQ  
ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14 and SEQ ID  
NO:18. Moreover, the present invention provides modified NPT genes with the DNA  
sequences according to SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9,  
20 SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:17.

#### Gene of Interest

The gene of interest contained in the expression vector according to the invention  
25 comprises a nucleotide sequence of any length which codes for a product of interest.  
The gene product or "product of interest" is generally a protein, polypeptide, peptide  
or fragment or derivative thereof. However, it may also be RNA or antisense RNA.  
The gene of interest may be present in its full length, in shortened form, as a fusion  
gene or as a labelled gene. It may be genomic DNA or preferably cDNA or  
30 corresponding fragments of fusions. The gene of interest may be the native gene  
sequence, or it may be mutated or otherwise modified. Such modifications include  
codon optimisations for adapting to a particular host cell and humanisation. The

gene of interest may, for example, code for a secreted, cytoplasmic, nuclear-located, membrane-bound or cell surface-bound polypeptide.

5 The term "nucleotide sequence" or "nucleic acid sequence" indicates an oligonucleotide, nucleotides, polynucleotides and fragments thereof as well as DNA or RNA of genomic or synthetic origin which occur as single or double strands and can represent the coding or non-coding strand of a gene. Nucleic acid sequences may be modified using standard techniques such as site-specific mutagenesis or PCR-mediated mutagenesis (e.g. described in Sambrook et al., 1989 or Ausubel et  
10 al., 1994).

By "coding" is meant the property or capacity of a specific sequence of nucleotides in a nucleic acid, for example a gene in a chromosome or an mRNA, to act as a matrix for the synthesis of other polymers and macromolecules such as for example rRNA,  
15 tRNA, mRNA, other RNA molecules, cDNA or polypeptides in a biological process. Accordingly, a gene codes for a protein if the desired protein is produced in a cell or another biological system by transcription and subsequent translation of the mRNA. Both the coding strand whose nucleotide sequence is identical to the mRNA sequence and is normally also given in sequence databanks, e.g. EMBL or GenBank,  
20 and also the non-coding strand of a gene or cDNA which acts as the matrix for transcription may be referred to as coding for a product or protein. A nucleic acid which codes for a protein also includes nucleic acids which have a different order of nucleotide sequence on the basis of the degenerate genetic code but result in the same amino acid sequence of the protein. Nucleic acid sequences which code for  
25 proteins may also contain introns.

The term cDNA denotes deoxyribonucleic acids which are prepared by reverse transcription and synthesis of the second DNA strand from a mRNA or other RNA produced from a gene. If the cDNA is present as a double stranded DNA molecule it  
30 contains both a coding and a non-coding strand.

The term intron denotes non-coding nucleotide sequences of any length. They occur naturally in numerous eukaryotic genes and are eliminated from a previously

transcribed mRNA precursor by a process known as splicing. This requires precise excision of the intron at the 5' and 3' ends and correct joining of the resulting mRNA ends so as to produce a mature processed mRNA with the correct reading frame for successful protein synthesis. Many of the splice donor and splice acceptor sites involved in this splicing process, i.e. the sequences located directly at the exon-intron or intron-exon interfaces, have been characterised by now. For an overview see Ohshima et al., 1987.

#### Protein/Product of Interest

Proteins/polypeptides with a biopharmaceutical significance include for example antibodies, enzymes, cytokines, lymphokines, adhesion molecules, receptors and the derivatives or fragments thereof, but are not restricted thereto. Generally, all polypeptides which act as agonists or antagonists and/or have therapeutic or diagnostic applications are of value.

The term "polypeptides" is used for amino acid sequences or proteins and refers to polymers of amino acids of any length. This term also includes proteins which have been modified post-translationally by reactions such as glycosylation, phosphorylation, acetylation or protein processing. The structure of the polypeptide may be modified, for example, by substitutions, deletions or insertions of amino acids and fusion with other proteins while retaining its biological activity. The term "polypeptides" thus also includes, for example, fusion proteins consisting of an immunoglobulin component, e.g. the Fc component, and a growth factor, e.g. an interleukin.

Examples of therapeutic proteins are insulin, insulin-like growth factor, human growth hormone (hGH) and other growth factors, tissue plasminogen activator (tPA), erythropoietin (EPO), cytokines, e.g. interleukines (IL) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18 interferon (IFN)-alpha, -beta, -gamma, -omega or -tau, tumour necrosis factor (TNF) such as TNF-alpha, beta or gamma, TRAIL, G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF. Other examples are monoclonal, polyclonal, multispecific and single chain

antibodies and fragments thereof such as for example Fab, Fab', F(ab')<sub>2</sub>, Fc and Fc' fragments, light (L) and heavy (H) immunoglobulin chains and the constant, variable or hypervariable regions thereof as well as Fv and Fd fragments (Chamov et al., 1999). The antibodies may be of human or non-human origin. Humanised and  
5 chimeric antibodies are also possible.

Fab fragments (fragment antigen binding = Fab) consist of the variable regions of both chains which are held together by the adjacent constant regions. They may be produced for example from conventional antibodies by treating with a protease such  
10 as papain or by DNA cloning. Other antibody fragments are F(ab')<sub>2</sub> fragments which can be produced by proteolytic digestion with pepsin.

By gene cloning it is also possible to prepare shortened antibody fragments which consist only of the variable regions of the heavy (VH) and light chain (VL). These are  
15 known as Fv fragments (fragment variable = fragment of the variable part). As covalent binding via the cystein groups of the constant chains is not possible in these Fv fragments, they are often stabilised by some other method. For this purpose the variable region of the heavy and light chains are often joined together by means of a short peptide fragment of about 10 to 30 amino acids, preferably 15 amino acids.  
20 This produces a single polypeptide chain in which VH and VL are joined together by a peptide linker. Such antibody fragments are also referred to as single chain Fv fragments (scFv). Examples of scFv antibodies are known and described, cf. for example Huston et al., 1988.

25 In past years various strategies have been developed for producing multimeric scFv derivatives. The intention is to produce recombinant antibodies with improved pharmacokinetic properties and increased binding avidity. In order to achieve the multimerisation of the scFv fragments they are produced as fusion proteins with multimerisation domains. The multimerisation domains may be, for example, the  
30 CH3 region of an IgG or helix structures ("coiled coil structures") such as the Leucine Zipper domains. In other strategies the interactions between the VH and VL regions of the scFv fragment are used for multimerisation (e.g. dia, tri- and pentabodies).

The term diabody is used in the art to denote a bivalent homodimeric scFv derivative. Shortening the peptide linker in the scFv molecule to 5 to 10 amino acids results in the formation of homodimers by superimposing VH/VL chains. The diabodies may additionally be stabilised by inserted disulphite bridges. Examples of diabodies can  
5 be found in the literature, e.g. in Perisic et al., 1994.

The term minibody is used in the art to denote a bivalent homodimeric scFv derivative. It consists of a fusion protein which contains the CH3 region of an immunoglobulin, preferably IgG, most preferably IgG1, as dimerisation region. This  
10 connects the scFv fragments by means of a hinge region, also of IgG, and a linker region. Examples of such minibodies are described by Hu et al., 1996.

The term triabody is used in the art to denote a trivalent homotrimeric scFv derivative (Kortt et al., 1997). The direct fusion of VH VL without the use of a linker sequence  
15 leads to the formation of trimers.

The fragments known in the art as mini antibodies which have a b-i, tri- or tetravalent structure are also derivatives of scFv fragments. The multimerisation is achieved by means of di, tri- or tetrameric coiled coil structures (Pack et al., 1993 and 1995;  
20 Lovejoy et al., 1993).

#### Gene which codes for a fluorescent protein

In another embodiment the expression vector according to the invention contains a  
25 gene coding for a fluorescent protein, preferably functionally linked to the gene of interest. Preferably, both genes are transcribed under the control of a single heterologous promoter so that the protein/ product of interest and the fluorescent protein are coded by a bicistronic mRNA. This makes it possible to identify cells which produce the protein/product of interest in large amounts, by means of the  
30 expression rate of the fluorescent protein.

The fluorescent protein may be, for example, a green, bluish-green, blue, yellow or other coloured fluorescent protein. One particular example is green fluorescent



protein (GFP) obtained from *Aequorea victoria* or *Renilla reniformis* and mutants developed from them; cf. for example Bennet et al., 1998; Chalfie et al., 1994; WO 01/04306 and the literature cited therein.

- 5 Other fluorescent proteins and genes coding for them are described in WO 00/34318, WO 00/34326, WO 00/34526 and WO 01/27150 which are incorporated herein by reference. These fluorescent proteins are fluorophores of non-bioluminescent organisms of the species *Anthozoa*, for example *Anemonia majano*, *Clavularia sp.*, *Zoanthus sp. I*, *Zoanthus sp. II*, *Discosoma striata*, *Discosoma sp. "red"*, *Discosoma*  
10 *sp. "green"*, *Discosoma sp. "Magenta"*, *Anemonia sulcata*.

- The fluorescent proteins used according to the invention contain in addition to the wild-type proteins natural or genetically engineered mutants and variants, fragments, derivatives or variants thereof which have for example been fused with other proteins  
15 or peptides. The mutations used may for example alter the excitation or emission spectrum, the formation of chromophores, the extinction coefficient or the stability of the protein. Moreover, the expression in mammalian cells or other species can be improved by codon optimisation. According to the invention the fluorescent protein may also be used in fusion with a selectable marker, preferably an amplifiable  
20 selectable marker such as dihydrofolate reductase (DHFR).

- The fluorescence emitted by the fluorescent proteins makes it possible to detect the proteins, e.g. by throughflow cytometry with a fluorescence-activated cell sorter (FACS) or by fluorescence microscopy.
- 25

### Other regulatory elements

The expression vector contains at least one heterologous promoter which allows expression of the gene of interest and preferably also of the fluorescent protein.

5

The term promoter denotes a polynucleotide sequence which allows and controls the transcription of the genes or sequences functionally connected therewith. A promoter contains recognition sequences for binding RNA polymerase and the initiation site for transcription (transcription initiation site). In order to express a desired sequence in a certain cell type or a host cell a suitable functional promoter must be chosen. The skilled man will be familiar with a variety of promoters from various sources, including constitutive, inducible and repressible promoters. They are deposited in databanks such as GenBank, for example, and may be obtained as separate elements or elements cloned within polynucleotide sequences from commercial or individual sources. In inducible promoters the activity of the promoter may be reduced or increased in response to a signal. One example of an inducible promoter is the tetracycline (tet) promoter. This contains tetracycline operator sequences (tetO) which can be induced by a tetracycline-regulated transactivator protein (tTA). In the presence of tetracycline the binding of tTA to tetO is inhibited. Examples of other inducible promoters are the jun, fos, metallothionein and heat shock promoter (see also Sambrook et al., 1989; Gossen et al., 1994).

Of the promoters which are particularly suitable for high expression in eukaryotes, there are for example the ubiquitin/S27a promoter of the hamster (WO 97/15664), SV 40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, the long terminal repeat region of Rous Sarcoma Virus, the early promoter of human Cytomegalovirus. Examples of other heterologous mammalian promoters are the actin, immunoglobulin or heat shock promoter(s).

A corresponding heterologous promoter can be functionally connected to other regulatory sequences in order to increase/regulate the transcription activity in an expression cassette.

For example, the promoter may be functionally linked to enhancer sequences in order to increase the transcriptional activity. For this, one or more enhancers and/or several copies of an enhancer sequence may be used, e.g. a CMV or SV40 enhancer. Accordingly, an expression vector according to the invention, in another embodiment, contains one or more enhancers/ enhancer sequences, preferably a CMV or SV40 enhancer.

The term enhancer denotes a polynucleotide sequence which in the *cis* location acts on the activity of a promoter and thus stimulates the transcription of a gene functionally connected to this promoter. Unlike promoters the effect of enhancers is independent of position and orientation and they can therefore be positioned in front of or behind a transcription unit, within an intron or even within the coding region. The enhancer may be located both in the immediate vicinity of the transcription unit and at a considerable distance from the promoter. It is also possible to have a physical and functional overlap with the promoter. The skilled man will be aware of a number of enhancers from various sources (and deposited in databanks such as GenBank, e.g. SV40 enhancers, CMV enhancers, polyoma enhancers, adenovirus enhancers) which are available as independent elements or elements cloned within polynucleotide sequences (e.g. deposited at the ATCC or from commercial and individual sources). A number of promoter sequences also contain enhancer sequences such as the frequently used CMV promoter. The human CMV enhancer is one of the strongest enhancers identified hitherto. One example of an inducible enhancer is the metallothionein enhancer, which can be stimulated by glucocorticoids or heavy metals.

Another possible modification is, for example, the introduction of multiple Sp1 binding sites. The promoter sequences may also be combined with regulatory sequences which allow control/regulation of the transcription activity. Thus, the promoter can be made repressible/ inducible. This can be done for example by linking to sequences which are binding sites for up- or down-regulating transcription factors. The above mentioned transcription factor Sp1, for example, has a positive effect on the transcription activity. Another example is the binding site for the activator protein AP1, which may act both positively and negatively on transcription. The activity of

AP1 can be controlled by all kinds of factors such as, for example, growth factors, cytokines and serum (Faisst et al., 1992 and references therein). The transcription efficiency can also be increased by changing the promoter sequence by the mutation (substitution, insertion or deletion) of one, two, three or more bases and then determining, in a reporter gene assay, whether this has increased the promoter activity.

Basically, the additional regulatory elements include heterologous promoters, enhancers, termination and polyadenylation signals and other expression control elements. Both inducible and constitutively regulatory sequences are known for the various cell types.

"Transcription-regulatory elements" generally comprise a promoter upstream of the gene sequence to be expressed, transcription initiation and termination sites and a polyadenylation signal.

The term "transcription initiation site" refers to a nucleic acid in the construct which corresponds to the first nucleic acid which is incorporated in the primary transcript, i.e. the mRNA precursor. The transcription initiation site may overlap with the promoter sequences.

The term "transcription termination site" refers to a nucleotide sequence which is normally at the 3' end of the gene of interest or of the gene section which is to be transcribed, and which brings about the termination of transcription by RNA polymerase.

The "polyadenylation signal" is a signal sequence which causes cleavage at a specific site at the 3' end of the eukaryotic mRNA and post-transcriptional incorporation of a sequence of about 100-200 adenine nucleotides (polyA tail) at the cleaved 3' end. The polyadenylation signal comprises the sequence AATAAA about 10-30 nucleotides upstream of the cleavage site and a sequence located downstream. Various polyadenylation elements are known such as tk polyA, SV40 late and early polyA or BGH polyA (described for example in US 5,122,458).

In a preferred embodiment of the present invention each transcription unit has a promoter or a promoter/enhancer element, a gene of interest and/or a marker gene as well as a transcription termination element. In another preferred embodiment the transcription unit contains two further translation regulatory units.

"Translation regulatory elements" comprise a translation initiation site (AUG), a stop codon and a polyA signal for each polypeptide to be expressed. For optimum expression it may be advisable to remove, add or change 5'- and/or 3'-untranslated regions of the nucleic acid sequence which is to be expressed, in order to eliminate any potentially unsuitable additional translation initiation codons or other sequences which might affect expression at the transcription or expression level. In order to promote expression, ribosomal consensus binding sites may alternatively be inserted immediately upstream of the start codon. In order to produce a secreted polypeptide the gene of interest usually contains a signal sequence which codes for a signal precursor peptide which transports the synthesised polypeptide to and through the ER membrane. The signal sequence is often but not always located at the amino terminus of the secreted protein and is cleaved by signal peptidases after the protein has been filtered through the ER membrane. The gene sequence will usually but not necessarily contain its own signal sequence. If the native signal sequence is not present a heterologous signal sequence may be introduced in known manner. Numerous signal sequences of this kind are known to the skilled man and deposited in sequence databanks such as GenBank and EMBL.

One important regulatory element according to the invention is the internal ribosomal entry site (IRES). The IRES element comprises a sequence which functionally activates the translation initiation independently of a 5'-terminal methylguanosinium cap (CAP structure) and the upstream gene and in an animal cell allows the translation of two cistrons (open reading frames) from a single transcript. The IRES element provides an independent ribosomal entry site for the translation of the open reading frame located immediately downstream. In contrast to bacterial mRNA which may be multicistronic, i.e. it may code for numerous different polypeptides or products which are translated one after the other by the mRNA, the majority of mRNAs from

animal cells are monocistronic and code for only one protein or product. In the case of a multicistronic transcript in a eukaryotic cell the translation would be initiated from the translation initiation site which was closest upstream and would be stopped by the first stop codon, after which the transcript would be released from the ribosome.

5 Thus, only the first polypeptide or product coded by the mRNA would be produced during translation. By contrast, a multicistronic transcript with an IRES element which is functionally linked to the second or subsequent open reading frame in the transcript allows subsequent translation of the open reading frame located downstream thereof, so that two or more polypeptides or products coded by the same  
10 transcript are produced in the eukaryotic cell.

The IRES element may be of various lengths and various origins and may originate, for example, from the encephalomyocarditis virus (EMCV) or other Picorna viruses. Various IRES sequences and their use in the construction of vectors are described in  
15 the literature, cf. for example Pelletier et al., 1988; Jang et al., 1989; Davies et al., 1992; Adam et al., 1991; Morgan et al., 1992; Sugimoto et al., 1994; Ramesh et al., 1996; Mosser et al., 1997.

The gene sequence located downstream is functionally linked to the 3' end of the  
20 IRES element, i.e. the spacing is selected so that the expression of the gene is unaffected or only marginally affected or has sufficient expression for the intended purpose. The optimum permissible distance between the IRES element and the start codon of the gene located downstream thereof for sufficient expression can be determined by simple experiments by varying the spacing and determining the  
25 expression rate as a function of the spacing using reporter gene assays.

By the measures described it is possible to obtain an optimum expression cassette which is of great value for the expression of heterologous gene products. An expression cassette obtained by means of one or more such measures is therefore a  
30 further subject of the invention.

Hamster-Ubiquitin/S27a Promoter

In another embodiment the expression vector according to the invention contains the ubiquitin/S27a promoter of the hamster, preferably functionally linked to the gene of interest and even more preferably functionally linked to the gene of interest and the gene which codes for a fluorescent protein.

5

The ubiquitin/S27a promoter of the hamster is a powerful homologous promoter which is described in WO 97/15664. Such a promoter preferably has at least one of the following features: GC-rich sequence area, Sp1 binding site, polypyrimidine element, absence of a TATA box. Particularly preferred is a promoter which has an Sp1 binding site but no TATA box. Also preferred is a promoter which is constitutively activated and in particular is equally active under serum-containing, low-serum and serum-free cell culture conditions. In another embodiment it is an inducible promoter, particularly a promoter which is activated by the removal of serum.

15

A particularly advantageous embodiment is a promoter with a nucleotide sequence as contained in Fig. 5 of WO 97/15664. Particularly preferred are promoter sequences which contain the sequence from position -161 to -45 of Fig. 5.

The promoters used in the examples of the present patent specification each contain a DNA molecule with the sequence from position 1923 to 2406 of SEQ ID NO:39 of the attached sequence listing. This sequence corresponds to the fragment -372 to +111 from Fig. 5 of WO 97/15664 and represents the preferred promoter, i.e. a preferred promoter should incorporate this sequence region. Another suitable promoter fragment contains the sequence from position 2134 to 2406 (corresponding to -161 to +111 in Fig. 5 of WO 97/15664). A promoter which contains only the sequence from position 2251 to 2406 is no longer functional (corresponds to position -45 to +111 in Fig. 5 of WO 97/15664). It is possible to extend the promoter sequence in the 5' direction starting from position 2134.

30

It is also possible to use functional subfragments of the complete hamster ubiquitin/S27a promoter sequence as well as functional mutants/variants of the complete sequence of subfragments thereof which have been modified, for example,

by substitution, insertion or deletion. Corresponding subfragments, mutants or variants are hereinafter also referred to as "modified promoters".

5 A modified promoter, optionally combined with other regulatory elements, preferably has a transcription activity which corresponds to that of the promoter fragment from position 1923 to 2406 of the nucleotide sequence given in SEQ ID NO:21 (-372 to +111 from Fig. 5 of WO 97/15664). A modified promoter proves to be useful for the purposes of the invention if it has a transcription activity which has at least 50%, preferably at least 80%, more preferably at least 90% and most preferably at least 10 100% of the activity of the 1923 to 2406 fragment (-372 to +111 fragment) in a comparative reporter gene assay. Particularly preferred are modified promoters which have a minimum sequence homology to the wild-type sequence SEQ ID NO:39 of the hamster ubiquitin/ S27a promoter of at least 80%, preferably at least 85%, preferably at least 90%, more preferably at least 95% and most preferably at least 15 97% and have a corresponding promoter activity in a comparative reporter gene assay.

In a corresponding comparative reporter gene assay the promoter fragments to be tested including the reference sequence are cloned in front of a promoterless reporter 20 gene which codes, for example for luciferase, secreted alkaline phosphatase or green fluorescent protein (GFP). These constructs (promoter sequence + reporter gene) are subsequently introduced into the test cells, e.g. CHO-DG44, by transfection and the induction of the reporter gene expression by the promoter fragment in question is determined by measuring the protein content of the reporter gene. A 25 corresponding test is found for example in Ausubel et al., Current Protocols in Molecular Biology, 1994, updated.

The promoter sequence of the hamster ubiquitin/S27a promoter and the modified promoters, which may also include, for example, the 5' untranslated region or 30 selected fragments thereof, and the coding region as well as the 3'-untranslated region of the ubiquitin/S27a gene or selected fragments thereof, may be obtained by a skilled man with a knowledge of the sequence described in WO 97/15664 using various standard methods as described for example in Sambrook et al., 1989;



Ausubel et al., 1994. Starting from the sequence described in WO 97/15664 a suitable fragment may be selected, for example, and an oligonucleotide probe containing the sequence of this fraction may be chemically synthesised. A probe of this kind may be used for example to clone the ubiquitin/S27a gene or the 5' untranslated region or other fragments thereof, for example by hybridisation from a library of the hamster genome. Using the reporter gene assay described above the skilled man is in a position to identify promoter-active fragments without any great effort and use them for the purposes of the present invention. The 5' untranslated region or special fragments thereof can easily be obtained by PCR amplification with corresponding primers from genomic DNA or a genomic library. Fragments of the 5' untranslated region may also be obtained by limited exonuclease III digestion from larger DNA fragments. Such DNA molecules may also be chemically synthesised or produced from chemically synthesised fragments by ligation.

Deletion, insertion and substitution mutants may be produced by "site-specific mutagenesis" and/or "PCR-based mutagenesis techniques". Corresponding methods are mentioned for example in Lottspeich and Zorbas 1998 Chapter 36.1 with further references.

By cross-hybridisation with probes from the 5' untranslated region of the hamster ubiquitin/S27a gene or from the S27a part of the hamster ubiquitin S27a gene or the 3'-untranslated region it is also possible to identify and isolate suitable promoter sequences from corresponding homologous genes of other, preferably mammalian species. Suitable techniques are described by way of example in Lottspeich and Zorbas 1998 Chapter 23. Genes are "homologous" for the purposes of the invention if their nucleotide sequence exhibits at least 70%, preferably at least 80%, preferably at least 90%, more preferably at least 95% and most preferably at least 97% conformity to the nucleotide sequence of the gene with which it is homologous.

Using the measures described above it is possible to obtain an optimised expression cassette which is highly valuable for the expression of heterologous gene products. An expression cassette obtained by one or more such measures is therefore a further object of the invention.

Preparation of expression vectors according to the invention

The expression vector according to the invention may theoretically be prepared by conventional methods known in the art, as described by Sambrook et al. (1989), for example. Sambrook also describes the functional components of a vector, e.g. suitable promoters (in addition to the hamster ubiquitin/S27a promoter), enhancers, termination and polyadenylation signals, antibiotic resistance genes, selectable markers, replication starting points and splicing signals. Conventional cloning vectors may be used to produce them, e.g. plasmids, bacteriophages, phagemids, cosmids or viral vectors such as baculovirus, retroviruses, adenoviruses, adeno-associated viruses and herpes simplex virus, as well as artificial chromosomes/mini chromosomes. The eukaryotic expression vectors typically also contain prokaryotic sequences such as, for example, replication origin and antibiotic resistance genes which allow replication and selection of the vector in bacteria. A number of eukaryotic expression vectors which contain multiple cloning sites for the introduction of a polynucleotide sequence are known and some may be obtained commercially from various companies such as Stratagene, La Jolla, CA, USA; Invitrogen, Carlsbad, CA, USA; Promega, Madison, WI, USA or BD Biosciences Clontech, Palo Alto, CA, USA.

The heterologous promoter, the gene of interest and the modified neomycin phosphotransferase gene and optionally the gene coding for a fluorescent protein, additional regulatory elements such as the internal ribosomal entry site (IRES), enhancers or a polyadenylation signal are introduced into the expression vector in a manner familiar to those skilled in the art. An expression vector according to the invention contains, at the minimum, a heterologous promoter, the gene of interest and a modified neomycin phosphotransferase gene. Preferably, the expression vector also contains a gene coding for a fluorescent protein. It is particularly preferred according to the invention to use a ubiquitin/S27a promoter as heterologous promoter. Particularly preferred is an expression vector in which the heterologous promoter, preferably a ubiquitin/S27a promoter, the gene of interest and the gene which codes for a fluorescent protein are functionally linked together or

are functionally linked and the neomycin phosphotransferase gene is located in the same or in a separate transcription unit.

Within the scope of the present description the term "functional linking" or "functionally linked" refers to two or more nucleic acid sequences or partial sequences which are positioned so that they can perform their intended function. For example, a promoter/enhancer is functionally linked to a coding gene sequence if it is able to control or modulate the transcription of the linked gene sequence in the cis position. Generally, but not necessarily, functionally linked DNA sequences are close together and, if two coding gene sequences are linked or in the case of a secretion signal sequence, in the same reading frame. Although a functionally linked promoter is generally located upstream of the coding gene sequence it does not necessarily have to be close to it. Enhancers need not be close by either, provided that they assist the transcription of the gene sequence. For this purpose they may be both upstream and downstream of the gene sequence, possibly at some distance from it. A polyadenylation site is functionally linked to a gene sequence if it is positioned at the 3' end of the gene sequence in such a way that the transcription progresses via the coding sequence to the polyadenylation signal. Linking may take place according to conventional recombinant methods, e.g. by the PCR technique, by ligation at suitable restriction cutting sites or by splicing. If no suitable restriction cutting sites are available synthetic oligonucleotide linkers or adaptors may be used in a manner known *per se*. According to the invention the functional linking preferably does not take place via intron sequences.

In one of the embodiments described, the heterologous promoter, preferably a ubiquitin/S27a promoter, the gene of interest and the gene coding for a fluorescent protein are functionally linked together. This means for example that both the gene of interest and the gene coding for a fluorescent protein are expressed starting from the same heterologous promoter.

In a particularly preferred embodiment the functional linking takes place via an IRES element, so that a bicistronic mRNA is synthesised from both genes. The expression vector according to the invention may additionally contain enhancer elements which

act functionally on one or more promoters. Particularly preferred is an expression vector in which the heterologous promoter, preferably the ubiquitin/S27a promoter or a modified form thereof, is linked to an enhancer element, e.g. an SV40 enhancer or a CMV enhancer element.

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Fundamentally, the expression of the genes within an expression vector may take place starting from one or more transcription units. The term transcription unit is defined as a region which contains one or more genes to be transcribed. The genes within a transcription unit are functionally linked to one another in such a way that all the genes within such a unit are under the transcriptional control of the same promoter or promoter/ enhancer. As a result of this transcriptional linking of genes, more than one protein or product can be transcribed from a transcription unit and thus expressed. Each transcription unit contains the regulatory elements which are necessary for the transcription and translation of the gene sequences contained therein. Each transcription unit may contain the same or different regulatory elements. IRES elements or introns may be used for the functional linking of the genes within a transcription unit.

The expression vector may contain a single transcription unit for expressing the gene of interest, the modified NPT gene and optionally the gene which codes for the fluorescent protein. Alternatively, these genes may also be arranged in two or more transcription units. Various combinations of the genes within a transcription unit are possible. In another embodiment of the present invention more than one expression vector consisting of one, two or more transcription units may be inserted in a host cell by cotransfection or in successive transfections in any desired order. Any combination of regulatory elements and genes on each vector can be selected provided that adequate expression of the transcription units is ensured. If necessary, other regulatory elements and genes, e.g. additional genes of interest or selectable markers, may be positioned on the expression vectors.

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Accordingly, an expression vector according to the invention containing a gene of interest and a gene which codes for a modified neomycin phosphotransferase may contain both genes in one or in two separate transcription units. Each transcription

unit can transcribe and express one or more gene products. If both genes are contained in one transcription unit they are under the control of the same promoter or promoter/ enhancer, while preferably an IRES element is used to ensure the functional linking of all the components. If the gene which codes for modified neomycin phosphotransferase and the gene of interest are contained in two separate transcription units, they may be under the control of the same or different promoters/enhancers. However, preferably, a weaker heterologous promoter, e.g. SV40 early promoter, is used for the modified NPT gene and preferably no enhancer is used. Expression vectors with two separate transcription units are preferred within the scope of the invention. One (bicistronic) transcription unit contains the gene of interest and optionally a gene coding for a fluorescent protein, while the other transcription unit contains the modified NPT gene. Preferably, each transcription unit is limited at the 3' end by a sequence which codes for a polyA signal, preferably BGH polyA or SV40 polyA.

Also preferred according to the invention are those expression vectors which instead of the gene of interest have only a multiple cloning site which allows the cloning of the gene of interest via recognition sequences for restriction endonucleases. Numerous recognition sequences for all kinds of restriction endonucleases as well as the associated restriction endonucleases are known from the prior art. Preferably, sequences are used which consist of at least six nucleotides as recognition sequence. A list of suitable recognition sequences can be found for example in Sambrook et al., 1989.

## Host Cells

For transfection with the expression vector according to the invention eukaryotic host cells are used, preferably mammalian cells and more particularly rodent cells such as mouse, rat and hamster cell lines. The successful transfection of the corresponding cells with an expression vector according to the invention results in transformed, genetically modified, recombinant or transgenic cells, which are also the subject of the present invention.

Preferred host cells for the purposes of the invention are hamster cells such as BHK21, BHK TK<sup>-</sup> CHO, CHO-K1, CHO-DUKX, CHO-DUKX B1 and CHO-DG44 cells or derivatives/descendants of these cell lines. Particularly preferred are CHO-DG44, CHO-DUKX, CHO-K1 and BHK21 cells, particularly CHO-DG44 and CHO-DUKX cells. Also suitable are myeloma cells from the mouse, preferably NS0 and Sp2/0 cells and derivatives/descendants of these cell lines.

Examples of hamster and mouse cells which can be used according to the invention are given in Table 1 that follows. However, derivatives and descendants of these cells, other mammalian cells including but not restricted to cell lines of humans, mice, rats, monkeys, rodents, or eukaryotic cells, including but not restricted to yeast, insect and plant cells, may also be used as host cells for the production of biopharmaceutical proteins.

Table 1: Hamster and Mouse Production Cell Lines

Cell line	Accession Number
NS0	ECASS No. 85110503
Sp2/0-Ag14	ATCC CRL-1581
BHK21	ATCC CCL-10
BHK TK <sup>-</sup>	ECACC No. 85011423
HaK	ATCC CCL-15
2254-62.2 (BHK-21-derivative)	ATCC CRL-8544
CHO	ECACC No. 8505302
CHO-K1	ATCC CCL-61
CHO-DUKX (= CHO duk <sup>-</sup> CHO/dhfr <sup>-</sup> )	ATCC CRL-9096
CHO-DUKX B1	ATCC CRL-9010
CHO-DG44	Urlaub et al; Cell 32[2], 405-412, 1983

CHO Pro-5	ATCC CRL-1781
V79	ATCC CCC-93
B14AF28-G3	ATCC CCL-14
CHL	ECACC No. 87111906

The transfection of the eukaryotic host cells with a polynucleotide or one of the expression vectors according to the invention is carried out by conventional methods (Sambrook et al., 1989; Ausubel et al., 1994). Suitable methods of transfection include for example lysosome-mediated transfection, calcium phosphate co precipitation, electroporation, polycation- (e.g. DEAE dextran)-mediated transfection, protoplast fusion, microinjection and viral infections. According to the invention stable transfection is preferably carried out in which the constructs are either integrated into the genome of the host cell or an artificial chromosome/minichromosome, or are episomally contained in stable manner in the host cell. The transfection method which gives the optimum transfection frequency and expression of the heterologous gene in the host cell in question is preferred. By definition, every sequence or every gene inserted in a host cell is referred to as a "heterologous sequence" or "heterologous gene" in relation to the host cell. This applies even if the sequence to be introduced or the gene to be introduced is identical to an endogenous sequence or an endogenous gene of the host cell. For example, a hamster actin gene introduced into a hamster host cell is by definition a heterologous gene.

According to the invention, recombinant mammalian cells, preferably rodent cells, most preferably hamster cells such as CHO or BHK cells which have been transfected with one of the expression vectors according to the invention described herein are preferred.

In the recombinant production of heteromeric proteins such as e.g. monoclonal antibodies (mAb), the transfection of suitable host cells can theoretically be carried out by two different methods. mAb's of this kind are composed of a number of subunits, the heavy and light chains. Genes coding for these subunits may be accommodated in independent or multicistronic transcription units on a single plasmid with which the host cell is then transfected. This is intended to secure the

stoichiometric representation of the genes after integration into the genome of the host cell. However, in the case of independent transcriptional units it must hereby be ensured that the mRNAs which encode the different proteins display the same stability and transcriptional and translational efficiency. In the second case, the expression of the genes take place within a multicistronic transcription unit by means of a single promoter and only one transcript is formed. By using IRES elements, a highly efficient internal translation initiation of the genes is obtained in the second and subsequent cistrons. However, the expression rates for these cistrons are lower than that of the first cistron, the translation initiation of which, by means of a so-called "cap"-dependent pre-initiation complex, is substantially more efficient than IRES-dependent translation initiation. In order to achieve a truly equimolar expression of the cistrons, additional inter-cistronic elements may be introduced, for example, which ensure uniform expression rates in conjunction with the IRES elements (WO 94/05785).

Another possible way of simultaneously producing a number of heterologous proteins, which is preferred according to the invention, is cotransfection, in which the genes are separately integrated in different expression vectors. This has the advantage that certain proportions of genes and gene products with one another can be adjusted, thereby balancing out any differences in the mRNA stability and in the efficiency of transcription and translation. In addition, the expression vectors are more stable because of their small size and are easier to handle both during cloning and during transfection.

In one particular embodiment of the invention, therefore, the host cells are additionally transfected, preferably cotransfected, with one or more vectors having genes which code for one or more other proteins of interest. The other vector or vectors used for the cotransfection code, for example, for the other protein or proteins of interest under the control of the same promoter/enhancer combination and for at least one other selectable marker, e.g. dihydrofolate reductase.

According to the invention the host cells are preferably established, adapted and cultivated under serum-free conditions, optionally in media which are free from animal



proteins/peptides. Examples of commercially obtainable media include Ham's F12 (Sigma, Deisenhofen, DE), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM; Sigma), Minimal Essential Medium (MEM; Sigma), Iscove's Modified Dulbecco's Medium (IMDM; Sigma), CD-CHO (Invitrogen, Carlsbad, CA, USA), CHO-S-SFMII (Invitrogen), serum-free CHO-Medium (Sigma) and protein-free CHO-Medium (Sigma). Each of these media may optionally be supplemented with various compounds, e.g. hormones and/or other growth factors (e.g. insulin, transferrin, epidermal growth factor, insulin-like growth factor), salts (e.g. sodium chloride, calcium, magnesium, phosphate), buffers (e.g. HEPES), nucleosides (e.g. adenosine, thymidine), glutamine, glucose or other equivalent nutrients, antibiotics and/or trace elements. Although serum-free media are preferred according to the invention, the host cells may also be cultivated and protein subsequently produced using media which have been mixed with a suitable amount of serum. In order to select genetically modified cells which express one or more selectable marker genes, one or more selecting agents are added to the medium.

The term "selecting agent" refers to a substance which affects the growth or survival of host cells with a deficiency for the selectable marker gene in question. Within the scope of the present invention, geneticin (G418) is preferably used as the medium additive for the selection of heterologous host cells which carry a modified neomycin phosphotransferase gene. Preferably, G418 concentrations of between 100 and 800 µg/ml of medium are used, most preferably 300 to 400 µg/ml of medium. If the host cells are to be transfected with a number of expression vectors, e.g. if several genes of interest are to be separately introduced into the host cell, they generally have different selectable marker genes.

A selectable marker gene is a gene which allows the specific selection of cells which contain this gene by the addition of a corresponding selecting agent to the cultivation medium. As an illustration, an antibiotic resistance gene may be used as a positive selectable marker. Only cells which have been transformed with this gene are able to grow in the presence of the corresponding antibiotic and are thus selected. Untransformed cells, on the other hand, are unable to grow or survive under these selection conditions. There are positive, negative and bifunctional selectable

markers. Positive selectable markers permit the selection and hence enrichment of transformed cells by conferring resistance to the selecting agent or by compensating for a metabolic or catabolic defect in the host cell. By contrast, cells which have received the gene for the selectable marker can be selectively eliminated by negative selectable markers. An example of this is the thymidine kinase gene of the Herpes Simplex virus, the expression of which in cells with the simultaneous addition of acyclovir or gancyclovir leads to the elimination thereof. The selectable markers used in this invention, including the amplifiable selectable markers, include genetically modified mutants and variants, fragments, functional equivalence, derivatives, homologues and fusions with other proteins or peptides, provided that the selectable marker retains its selective qualities. Such derivatives display considerable homology in the amino acid sequence in the regions or domains which are deemed to be selective. The literature describes a large number of selectable marker genes including bifunctional (positive/negative) markers (see for example WO 92/08796 and WO 94/28143). Examples of selectable markers which are usually used in eukaryotic cells include the genes for aminoglycoside phosphotransferase (APH), hygromycin phosphotransferase (HYG), dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase, asparagin synthetase and genes which confer resistance to neomycin (G418), puromycin, histidinol D, belomycin, phleomycin and zeocin.

It is also possible to select transformed cells by fluorescence-activated cell sorting (FACS). For this, bacterial  $\beta$ -galactosidase, cell surface markers or fluorescent proteins may be used (e.g. green fluorescent protein (GFP) and the variants thereof from *Aequorea victoria* and *Renilla reniformis* or other species; red fluorescent protein and proteins which fluoresce in other colours and their variants from non-bioluminescent organisms such as e.g. *Discosoma sp.*, *Anemonia sp.*, *Clavularia sp.*, *Zoanthus sp.*) for the selection of transformed cells.

### Gene expression and selection of high-producing host cells

The term gene expression relates to the transcription and/or translation of a heterologous gene sequence in a host cell. The expression rate can be generally

determined, either on the basis of the quantity of corresponding mRNA which is present in the host cell or on the basis of the quantity of gene product produced which is encoded by the gene of interest. The quantity of mRNA produced by transcription of a selected nucleotide sequence can be determined for example by northern blot hybridisation, ribonuclease-RNA-protection, *in situ* hybridisation of cellular RNA or by PCR methods (Sambrook et al., 1989; Ausubel et al., 1994). Proteins which are encoded by a selected nucleotide sequence can also be determined by various methods such as, for example, ELISA, western blot, radioimmunoassay, immunoprecipitation, detection of the biological activity of the protein or by immune staining of the protein followed by FACS analysis (Sambrook et al., 1989; Ausubel et al., 1994).

The terms "high expression level (or rate), high expression, increased expression or high productivity" refer to the long-lasting and sufficiently high expression or synthesis of a heterologous sequence introduced into a host cell, e.g. of a gene coding for a therapeutic protein. Increased or high expression or a high expression level or rate or a high productivity are present if a cell according to the invention is cultivated by one of the methods according to the invention described here, without gene amplification, and if this cell produces at least more than roughly 0.5 pg of the desired gene product per day (0.5 pg/day/cell). Increased or high expression or a high expression or rate or a high productivity are also present if the cell according to the invention without prior gene amplification produces at least more than roughly 1.0 pg of the desired gene product per day (1.0 pg/day/cell). Increased or high expression or a high expression level or rate or high productivity are present in particular if the cell according to the invention without prior gene amplification produces at least more than roughly 1.5 pg of the desired gene product per day (1.5 pg/day/cell). Increased or high expression or a high expression level or rate or high productivity are present in particular if the cell according to the invention without prior gene amplification produces at least more than roughly 2.0 pg of the desired gene product per day (2.0 pg/day/cell). Particularly increased or high expression or a particularly high expression level or rate or particularly high productivity are present if the cell according to the invention without prior gene amplification produces at least more than roughly 3.0 pg of the desired gene product per day (3.0 pg/day/cell). By

means of a simple gene amplification step, e.g. using the DHFR/MTX amplification system as described hereinafter the productivities can be increased by a factor of at least 2 to 10, so that the terms "high expression", "increased expression" or "high productivity" are used in relation to a cell which has been subjected to a gene amplification step if this cell produces at least more than roughly 5 pg of the desired gene product per day (5 pg/day/cell), preferably at least more than roughly 10 pg/day/cell, more preferably at least more than roughly 15 pg/day/cell, still more preferably at least more than roughly 20 pg/day/cell or at least more than roughly 30 pg/day/cell.

High or increased expression, high productivity or a high expression level or rate can be achieved both by using one of the expression vectors according to the invention and by the use of one of the processes according to the invention.

For example, by coexpression of the gene of interest and a modified NPT gene it is possible to select and identify cells which express the heterologous gene to a high degree. Compared with wtNPT, modified NPT allows more efficient selection of stably transfected host cells with high expression of the heterologous gene of interest.

The present invention thus also relates to a process for expressing at least one gene of interest in recombinant mammalian cells, characterised in that (i) a pool of mammalian cells is transfected with at least one gene of interest and one gene for a modified neomycin phosphotransferase which compared with the wild-type neomycin phosphotransferase has only 1 to 80% of the activity, preferably only 1 to 60%, more preferably only 1.5 to 30%, most preferably only 1.5 to 26%; (ii) the cells are cultivated under conditions which allow expression of the gene or genes of interest and the modified neomycin phosphotransferase gene; (iii) the mammalian cells are cultivated in the presence of at least one selecting agent, preferably G418, which acts selectively on the growth of the mammalian cells, and gives preference to the growth of those cells which express the modified neomycin phosphotransferase gene; and (iv) the protein or proteins of interest is or are obtained from the mammalian cells or the culture supernatant. Preferably recombinant mammalian

cells are used which have been transfected with an expression vector according to the invention.

5 The invention also relates to a process for selecting recombinant mammalian cells which express at least one gene of interest, wherein (i) a pool of mammalian cells is transfected with at least one gene of interest and a gene for a modified neomycin phosphotransferase which by comparison with wild-type neomycin phosphotransferase has only 1 to 80% of the activity, preferably only 1 to 60%, more preferably only 1.5 to 30%, most preferably only 1.5 to 26%; (ii) the mammalian cells  
10 are cultivated under conditions which allow expression of the gene or genes of interest and the modified neomycin phosphotransferase gene; and (iii) the mammalian cells are cultivated in the presence of at least one selecting agent, preferably G418, which acts selectively on the growth of the mammalian cells and gives preference to the growth of those cells which express the modified neomycin  
15 phosphotransferase gene.

Particularly preferred are processes for expressing at least one gene of interest and for selecting recombinant cells which express a corresponding gene of interest if a modified NPT gene described in more detail in this application is used, particularly if a  
20 modified NPT gene is used which by comparison with the wild-type gene codes for glycine at amino acid position 182, for alanine at amino acid position 91, for glycine at amino acid position 198, for alanine or valine at amino acid position 227, for glycine or asparagine at amino acid position 261 or for isoleucine at amino acid position 240. It is particularly preferred to use the Asp227Val, Asp261Gly,  
25 Asp261Asn, Phe240Ile or Trp91Ala mutant. Generally, all the modified neomycin phosphotransferase genes according to the invention mentioned in this patent specification are suitable for such a process. For the preferred neomycin phosphotransferase genes see the section on modified neomycin phosphotransferase genes.

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The selection of the cells which express a gene of interest and a modified NPT gene is carried out for example by adding G418 as selecting agent. However, it is also possible to use other aminoglycoside antibiotics such as neomycin or kanamycin.

The cells according to the invention are preferably cultivated and selected in 200 to 800 µg of G418 per mL of culture medium. It has proved particularly preferable to add 300 to 700 µg of G418 per mL of culture medium. The addition of roughly 400 µg of G418 per mL of culture medium is the most preferred embodiment. Using such a method it is possible to select recombinant cells with a particularly high expression rate. By comparison with the use of wtNPT, after selection with 400 µg G418 per ml of culture medium as selectable marker, the cells exhibited a productivity increased by a factor of 1.4 in the case of the Glu182Gly and Val198Gly mutant, by a factor of 2.2 or 4 in the case of the Asp227Ala or Trp91Ala mutant, by a factor of 5.7 or 7.3 in the case of the Phe240Ile or Asp261Asn mutant and even by a factor of 9.3 or 14.6 in the case of the Asp261Gly or Asp227Val mutant. The specific productivities for the various modified NPT genes are shown in Fig. 5.

The corresponding processes may be combined with a FACS-assisted selection of recombinant host cells which contain, as additional selectable marker, one or more fluorescent proteins (e.g. GFP) or a cell surface marker. Other methods of obtaining increased expression, and a combination of different methods may also be used, are based for example on the use of (artificial) transcription factors, treatment of the cells with natural or synthetic agents for up-regulating endogenous or heterologous gene expression, improving the stability (half-life) of mRNA or the protein, improving the initiation of mRNA translation, increasing the gene dose by the use of episomal plasmids (based on the use of viral sequences as replication origins, e.g. SV40, polyoma, adenovirus, EBV or BPV), the use of amplification-promoting sequences (Hemann et al., 1994) or in vitro amplification systems based on DNA concatemers (Monaco et al., 1996).

Coupled transcription of the gene of interest and the gene which codes for the fluorescent protein has proved particularly effective in conjunction with the use of a modified NPT gene as selectable marker. The resulting bicistronic mRNA expresses both the protein/product of interest and the fluorescent protein. On the basis of this coupling of the expression of the protein of interest and the fluorescent protein it is easily possible according to the invention to identify and isolate high-producing

recombinant host cells by means of the fluorescent protein expressed, e.g. by sorting using fluorescence activated cell sorting equipment (FACS).

5 The selection of recombinant host cells which exhibit high vitality and an increased expression rate of the desired gene product is a multistage process. The host cells which have been transfected with the expression vector according to the invention or optionally cotransfected with another vector, for example, are cultivated under conditions which permit the selection of cells expressing the modified NPT, e.g. by cultivation in the presence of a selecting agent such as G418 in concentrations of  
10 100, 200, 400, 600, 800 µg or more of G418/mL of culture medium. Then the corresponding cells are investigated at least for the expression of the gene which codes for a fluorescent protein and is coupled to the gene of interest, in order to identify and sort out the cells/cell population which exhibit the highest expression rates of fluorescent protein. Preferably, only the cells which belong to the 10% of  
15 cells with the highest expression rate of fluorescent protein are sorted out and further cultivated. In practice this means that the brightest 10% of the fluorescent cells are sorted out and further cultivated. Accordingly, the brightest 5%, preferably the brightest 3% or even the brightest 1% of the fluorescent cells of a cell mixture can also be sorted out and replicated. In a particularly preferred embodiment only the  
20 brightest 0.5% or the brightest 0.1% of the fluorescent cells are sorted out and replicated.

The selection step may be carried out on cell pools or using pre-sorted cell pools/cell clones. One or more, preferably two or more and especially three or more sorting  
25 steps may be carried out, while between the individual sorting steps the cells may be cultivated and replicated for a specific length of time, e.g. roughly two weeks in the case of pools.

The present invention thus relates to a process for obtaining and selecting  
30 recombinant mammalian cells which express at least one heterologous gene of interest, characterised in that (i) recombinant mammalian cells are transfected with an expression vector according to the invention; (ii) the transfected cells are cultivated under conditions which allow expression of the gene or genes of interest,

the gene coding for a fluorescent protein and the modified neomycin phosphotransferase gene; (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells and gives preference to the growth of those cells which express the modified  
5 neomycin phosphotransferase gene; and (iv) the mammalian cells which exhibit a particularly high expression of the fluorescent gene are sorted out by flow-cytometric analysis. If desired steps (ii) to (iv) may be repeated once or several times with the cells obtained in step (iv).

- 10 A corresponding process is preferred which is characterised in that the sorted mammalian cells have an average specific productivity, without an additional gene amplification step, of more than 0.5 pg of the desired gene product or products per day and per cell (5 pg/day/cell), preferably greater than 1.0 pg/day/cell, more preferably greater than 2.0 pg/day/cell. As mentioned above, the productivity of  
15 these cells can be increased by a simple gene amplification step, e.g. using the DHFR/MTX system, by a factor of at least 2 to 10.

- Also preferred according to the invention is a process in which suitably sorted cells are replicated and used to prepare the encoded gene product of interest. For this,  
20 the selected high producing cells are preferably cultivated in a serum-free culture medium and preferably in suspension culture under conditions which allow expression of the gene of interest. The protein/product of interest is preferably obtained from the cell culture medium as a secreted gene product. If the protein is expressed without a secretion signal, however, the gene product may also be  
25 isolated from cell lysates. In order to obtain a pure homogeneous product which is substantially free from other recombinant proteins and host cell proteins, conventional purification procedures are carried out. First of all, cells and cell debris are removed from the culture medium or lysate. The desired gene product can then be freed from contaminating soluble proteins, polypeptides and nucleic acids, e.g. by fractionation  
30 on immunoaffinity and ion exchange columns, ethanol precipitation, reversed phase HPLC or chromatography on Sephadex, silica or cation exchange resins such as DEAE. Methods which result in the purification of a heterologous protein expressed



by recombinant host cells are known to the skilled man and described in the literature, e.g. by Harris et al., 1995 and Scopes 1988.

### Amplifiable Selectable Marker Gene

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In addition, the cells according to the invention may optionally also be subjected to one or more gene amplification steps in which they are cultivated in the presence of a selecting agent which leads to amplification of an amplifiable selectable marker gene. This step may be carried out both with cells which express a fluorescent protein and have preferably been pre-sorted once or several times by FACS (preferably in one of the ways described here) and with cells which have not yet been sorted.

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The prerequisite is that the host cells are additionally transfected with a gene which codes for an amplifiable selectable marker. It is conceivable for the gene which codes for an amplifiable selectable marker to be present on one of the expression vectors according to the invention or to be introduced into the host cell by means of another vector.

20

The amplifiable selectable marker gene usually codes for an enzyme which is needed for the growth of eukaryotic cells under certain cultivation conditions. For example, the amplifiable selectable marker gene may code for dihydrofolate reductase (DHFR). In this case the gene is amplified if a host cell transfected therewith is cultivated in the presence of the selecting agent methotrexate (MTX).

25

The following Table 2 gives examples of other amplifiable selectable marker genes and the associated selecting agents which may be used according to the invention, which are described in an overview by Kaufman, Methods in Enzymology, 185:537-566 (1990).

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Table 2: Amplifiable selectable marker genes

Amplifiable selectable marker gene	Accession number	Selecting agent
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dihydrofolate reductase	M19869 (hamster) E00236 (mouse)	methotrexate (MTX)
metallothionein	D10551 (hamster) M13003 (human) M11794 (rat)	cadmium
CAD (carbamoylphosphate synthetase : aspartate transcarbamylase: dihydroorotase)	M23652 (hamster) D78586 (human)	N-phosphoacetyl-L-aspartate
adenosine-deaminase	K02567 (human) M10319 (mouse)	Xyl-A- or adenosine, 2'-deoxycoformycin
AMP (adenylate)-deaminase	D12775 (human) J02811 (rat)	adenine, azaserin, coformycin
UMP-synthase	J03626 (human)	6-azauridine, pyrazofuran
IMP 5'-dehydrogenase	J04209 (hamster) J04208 (human) M33934 (mouse)	mycophenolic acid
xanthine-guanin-phosphoribosyltransferase	X00221 (E. coli)	mycophenolic acid with limiting xanthine
mutant HGPRTase or mutant thymidine-kinase	J00060 (hamster) M13542, K02581 (human) J00423, M68489(mouse) M63983 (rat) M36160 (Herpes virus)	hypoxanthine, aminopterin and thymidine (HAT)
thymidylate-synthetase	D00596 (human) M13019 (mouse) L12138 (rat)	5-fluorodeoxyuridine
P-glycoprotein 170 (MDR1)	AF016535 (human) J03398 (mouse)	several drugs, e.g. adriamycin, vincristin, colchicine
ribonucleotide reductase	M124223, K02927 (mouse)	aphidicoline
glutamine-synthetase	AF150961 (hamster) U09114, M60803 (mouse) M29579 (rat)	methionine sulfoximine (MSX)
asparagine-synthetase	M27838 (hamster) M27396 (human) U38940 (mouse) U07202 (rat)	$\beta$ -aspartylhydroxamate, albizziin, 5'-azacytidine
argininosuccinate-synthetase	X01630 (human) M31690 (mouse) M26198 (bovine)	canavanin
ornithine-decarboxylase	M34158 (human) J03733 (mouse) M16982 (rat)	$\alpha$ -difluoromethylornithine

HMG-CoA-reductase	L00183,M12705 (hamster) M11058 (human)	compactin
N-acetylglucosaminyl-transferase	M55621 (human)	tunicamycin
threonyl-tRNA-synthetase	M63180 (human)	borrelidin
Na <sup>+</sup> K <sup>+</sup> -ATPase	J05096 (human) M14511 (rat)	ouabain

According to the invention the amplifiable selectable marker gene used is preferably a gene which codes for a polypeptide with the function of DHFR, e.g. for DHFR or a fusion protein from the fluorescent protein and DHFR. DHFR is necessary for the biosynthesis of purines. Cells which lack the DHFR genes cannot grow in purine-deficient medium. The DHFR gene is therefore a useful selectable marker for selecting and amplifying genes in cells cultivated in purine-free medium. The selecting medium used in conjunction with the DHFR gene is methotrexate (MTX).

The present invention therefore includes a method of preparing and selecting recombinant mammalian cells which contains the following steps: (i) transfection of the host cells with genes which code at least for a protein/ product of interest, a modified neomycin phosphotransferase and DHFR; (ii) cultivation of the cells under conditions which allow expression of the various genes; and (iii) the amplification of the co-integrated genes by cultivating the cells in the presence of a selecting agent which allows the amplification of at least the amplifiable selectable marker gene such as methotrexate. Preferably the transfected cells are cultivated in hypoxanthine/thymidine-free medium in the absence of serum and with the addition of increasing concentrations of MTX. Preferably the concentration of MTX in the first amplification step is at least 5 nM. The concentration of MTX may, however, also be at least 20 nM or 100 nM and be increased step by step to 1 µM. In individual cases concentrations of more than 1 µM may be used, e.g. 2 µM.

If the corresponding cells are additionally transformed with a gene for a fluorescent protein, these cells may be identified and sorted using a fluorescence activated cell sorting device (FACS) and then cultivated in a gene amplification step in the presence of at least 20, preferably in the presence of 50 or 100 nM MTX. In this way

it is possible to increase productivities substantially to more than 20 pg of gene product per cell and per day. The host cells may be subjected to one or more gene amplification steps in order to increase the copy number of at least the gene of interest and the amplifiable selectable marker gene. According to the invention the high productivity which can be achieved is linked to effective pre-selection by means of neomycin phosphotransferase- mediated resistance to aminoglycoside antibiotics such as neomycin, kanamycin and G418. It is therefore possible to reduce the number of gene amplification steps required and to carry out only a single gene amplification, for example.

In a further embodiment the present invention thus also relates to processes for obtaining and selecting recombinant mammalian cells which express at least one heterologous gene of interest and are characterised in that (i) recombinant mammalian cells are transfected with an expression vector according to the invention and the gene for an amplifiable selectable marker gene; (ii) the mammalian cells are cultivated under conditions which allow expression of the gene or genes of interest, the modified neomycin phosphotransferase gene and the gene which codes for a fluorescent protein; (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells and gives preference to the growth of those cells which express the neomycin phosphotransferase gene; (iv) the mammalian cells which exhibit high expression of the fluorescent protein are sorted out by flow-cytometric analysis; (v) the sorted cells are cultivated under conditions under which the amplifiable selectable marker gene is expressed; and (vi) a selecting agent is added to the culture medium which results in the amplification of the amplifiable selectable marker gene.

Particularly preferred is a corresponding process in which the modified neomycin phosphotransferase genes described in this invention are used. Also preferred is a process in which only one amplification step is carried out. Also preferred is a corresponding process which leads to recombinant mammalian cells which exhibit an average specific productivity of more than 20 pg of the desired gene product or products per cell and per day.

Mammalian cells, preferably mouse myeloma and hamster cells, are preferred host cells for the use of DHFR as an amplifiable selectable marker. The cell lines CHO-DUKX (ATCC CRL-9096) and CHO-GD44 (Urlaub et al., 1983) are particularly preferred as they have no DHFR activity of their own, as a result of mutation. In order to be able to use the DHFR-induced amplification in other cell types as well which have their own endogenous DHFR activity, it is possible to use in the transfection process a mutated DHFR gene which codes for a protein with reduced sensitivity to methotrexate (Simonson et al., 1983; Wigler et al., 1980; Haber et al., 1982).

The DHFR marker is particularly suitable for the selection and subsequent amplification when using DHFR negative basic cells such as CHO-DG44 or CHO-DUKX, as these cells do not express endogenous DHFR and therefore do not grow in purine-free medium. Consequently, the DHFR gene may be used here as a dominant selectable marker and the transformed cells are selected in hypoxanthine/thymidine-free medium.

The invention is described more fully hereinafter with reference to some non-restrictive examples.

## EXAMPLES

### Abbreviations

Ala (=A)	alanine
AP:	alkaline phosphatase
Asn (=N)	asparagine
Asp (=D):	aspartic acid
bp:	base pair
BSA:	bovine serum albumin
CHO:	Chinese Hamster Ovary
dhfr:	dihydrofolate-reductase

	DMSO:	dimethylsulphoxide
	ELISA:	enzyme-linked immunosorbent assay
	FACS:	fluorescence-activated cell sorter
	FITC:	fluoresceine-isothiocyanate
5	GFP:	green fluorescent protein
	Glu (=E):	glutamic acid
	Gly (=G):	glycine
	HBSS:	Hanks Balanced Salt Solution
	HT:	hypoxanthine/thymidine
10	Ile (=I):	isoleucine
	IRES:	internal ribosomal entry site
	kb:	kilobase
	mAb:	monoclonal antibody
	MCP-1:	monocyte chemoattractant protein-1
15	MTX:	methotrexate
	MW:	mean value
	NPT:	neomycin-phosphotransferase
	PCR:	polymerase chain reaction
	PBS:	phosphate buffered saline
20	Phe (=F):	phenylalanine
	Trp (=W):	tryptophan
	Val (=V):	valine
	WT:	wild-type

## 25 Methods

### 1. Cell culture and Transfection

The cells CHO-DG44/dhfr<sup>-/-</sup> (Urlaub et al., 1983) were permanently cultivated as suspension cells in serum-free CHO-S-SFMII medium supplemented with  
30 hypoxanthine and thymidine (Invitrogen GmbH, Karlsruhe, DE) in cell culture flasks at 37°C in a damp atmosphere and 5% CO<sub>2</sub>. The cell counts and viability were determined with a CASY1 Cell Counter (Schaerfe System, DE) or by trypan blue

staining and the cells were then seeded in a concentration of  $1 - 3 \times 10^5/\text{mL}$  and run every 2 – 3 days.

Lipofectamine Plus Reagent (Invitrogen GmbH) was used for the transfection of CHO-DG44. For each transfection mixture a total of 1 µg of plasmid-DNA, 4 µL of lipofectamine and 6 µL of Plus reagent were mixed together according to the manufacturer's instructions and added in a volume of 200 µL to  $6 \times 10^5$  exponentially growing CHO-DG44 cells in 0.8 mL of HT-supplemented CHO-S-SFMII medium. After three hours' incubation at 37°C in a cell incubator 2 mL of HT-supplemented CHO-S-SFMII medium was added. For selection the cells were transferred 2 days after transfection into CHO-S-SFMII medium without the addition of hypoxanthine and thymidine and G418 (Invitrogen) was also added to the medium in a concentration of 400 µg/mL changing the medium every 3 to 4 days for the first 2 to 3 weeks.

A DHFR-based gene amplification of the integrated heterologous genes can be obtained by the addition of the selecting agent MTX (Sigma, Deisenhofen, DE) in a concentration of 5 – 2000 nM to an HT-free CHO-S-SFMII medium.

## 2. Expression vectors

To analyse the expression, eukaryotic expression vectors were used which are based on the pAD-CMV vector (Werner et al., 1998) and mediate the constitutive expression of a heterologous gene by the combination of CMV enhancer/hamster ubiquitin/S27a promoter (WO 97/15664). While the base vector pBID contains the dhfr-minigene which acts as an amplifiable selectable marker (cf e.g. EP-0-393-438), in the vector pBIN the dhfr-minigene has been replaced by a neomycin-phosphotransferase resistance gene (Fig.1). For this purpose the selectable marker neomycin-phosphotransferase, including SV40 early promoter and TK-polyadenylation signal, was isolated from the commercial plasmid pBK-CMV (Stratagene, La Jolla, CA, USA) as a 1640 bp Bsu36I fragment. After a reaction to fill in the ends of the fragment with Klenow-DNA-polymerase the fragment was ligated with the 3750 bp Bsu36I/StuI fragment of the vector pBID, which was also treated with Klenow-DNA-polymerase.

In the bicistronic base vector pBIDG (Fig. 1) the IRES-GFP gene region was isolated from the vector pIRES2-EGFP (Clontech, Palo Alto, CA, USA) and brought under the control of the CMV enhancer/promoter in the vector pBID so that the multiple cloning

site between the promoter region and IRES-element was retained. The following procedure was used. In a PCR mutagenesis in which the plasmid pIRES2-EGFP acted as the template, on the one hand the HindIII cutting site AAGCTT within the IRES sequence was converted into the sequence ATGCTT by the use of mutagenic primers and thus eliminated. On the other hand an XbaI cutting site was inserted by means of a primer with complementarity to the 5' end of the IRES sequence or a SpeI cutting site was introduced by means of a primer with complementarity to the 3' end of the GFP sequence. The resulting PCR fragment, which contained the complete IRES and GFP sequence, was digested with XbaI and SpeI and cloned into the singular XbaI cutting site at the 3' end of the multiple cloning site of the vector pBID. In the same way the IRES-GFP gene region from the vector pIRES2-EGFP was brought under the control of the CMV enhancer/hamster ubiquitin/S27a promoter in the vector pBIN. This produced the bicistronic base vector pBING (Fig.1).

In order to express a monoclonal humanised IgG2 antibody the heavy chain was cloned as a 1.5 kb BamHI/HindIII fragment into the vector pBIDG digested with BamHI and HindIII, to obtain the vector pBIDG-HC (Fig. 2). The light chain on the other hand was cloned as a 0.7 kb BamHI/HindIII fragment into the corresponding cutting sites of the vector pBIN, producing the vector pBIN-LC (Fig. 2).

### 3. FACS

The flow-cytometric analyses and sorting were carried out with a Coulter Epics Altra device. The FACS is fitted with a helium-argon laser with an excitation wavelength of 488 nm. The fluorescence intensity is absorbed at a wavelength suited to the fluorescence protein and process by means of the attached software Coulter Expo32. The sorting is normally carried out at a rate of 8000 – 10000 events/second. The suspended cells can be centrifuged (5 min at 180xg) and adjusted to a cell concentration of  $1 - 1.5 \times 10^7$ /mL in HBSS. Then the cells can be sorted according to their fluorescence protein signal. The cells are taken up in test tubes already containing culture medium, then centrifuged and, depending on the number of cells sorted, seeded into suitable culture vessels or deposited directly in microtitre plates.

### 4. ELISA



The IgG2 mAb in the supernatants from stably transfected CHO-DG44 cells was quantified by ELISA according to standard procedures (Current Protocols in Molecular Biology, Ausubel et al., 1994, updated), using on the one hand a goat anti human IgG Fc fragment (Dianova, Hamburg, DE) and on the other hand an AP-conjugated goat anti human kappa light chain antibody (Sigma). Purified IgG2 antibody was used as the standard.

Productivities (pg/cell/day) were calculated by the formula  $\text{pg}/((\text{Ct}-\text{Co}) \cdot t / \ln (\text{Ct}-\text{Co}))$ , where Co and Ct are the cell count on seeding and harvest, respectively, and t is the cultivation time.

#### 5. Dot Assay for determining the NPT enzyme activity

In order to prepare a cell extract according to a method of Duch et al., 1990,  $6 \times 10^6$  cells were washed twice with PBS and then resuspended in 600  $\mu\text{L}$  of extraction buffer (0.135 M Tris-HCl pH 6.8, 20% glycerol, 4 mM dithiothreitol). After four cycles of freezing and thawing in a bath of dry ice or water the cell debris was removed by centrifuging and the supernatant was used for the subsequent enzyme assay. The protein concentration in the cell extracts was determined by a Bradford assay using the BIO-RAD protein assay (Bio-Rad Laboratories GmbH, Munich, DE), with BSA as the standard protein (Current Protocols in Molecular Biology, Ausubel et al., 1994, updated). In order to determine the NPT enzyme activity a Dot Assay was carried out, based on the protocol of Platt et al. 1987. For this, 5  $\mu\text{g}$ , 2.5  $\mu\text{g}$  and 1.25  $\mu\text{g}$  of protein were adjusted with extraction buffer to a final volume of 20  $\mu\text{L}$ , topping up to a total protein content of 5  $\mu\text{g}$  with cell extract from non-transfected CHO-DG44 cells. After the addition of 200  $\mu\text{L}$  of assay buffer (67 mM Tris-HCl pH 7.1, 42 mM  $\text{MgCl}_2$ , 400 mM  $\text{NH}_4\text{Cl}$ ) plus/minus 40  $\mu\text{g}/\text{mL}$  G418 and plus/minus 5  $\mu\text{Ci}$   $[\gamma\text{-}^{33}\text{P}]\text{-ATP}/\text{mL}$  (NEN) the extracts were incubated at 27°C for 135 minutes. Then the extracts were filtered in a 96 well vacuum manifold (Schleicher & Schüll, Dassel, DE) through a sandwich of one layer of Whatman 3MM paper, P81 phosphocellulose membrane (Whatman Laboratory Division, Maidstone, Great Britain) and nitrocellulose membrane (Schleicher & Schüll). Proteins phosphorylated by protein kinases and non-phosphorylated proteins bind to the nitrocellulose, while phosphorylated G418 passes through the nitrocellulose and binds to the phosphocellulose. After washing three times with deionised  $\text{H}_2\text{O}$  the membranes were removed from the apparatus,

washed again with H<sub>2</sub>O and then air-dried. The radioactive signals were quantified using a Phospho Imager (Molecular Dynamics, Krefeld, DE).

#### 6. Northern Blot Analysis

5 Total RNA was isolated from the cells with the TRIZOL reagent according to the manufacturer's instructions (Invitrogen GmbH, Karlsruhe, DE) and the separation of 30 µg RNA by gel electrophoresis and the transfer to a Hybond N+ nylon membrane (Amersham Biosciences, Freiburg, DE) were carried out according to the standard procedure for glyoxal/DMSO-denatured RNA (Current Protocols in Molecular Biology, 10 Ausubel et al., 1994, updated). The probe used for the subsequent non-radioactive hybridisation with the GenelImages CDP-Star Detection Kit (Amersham Biosciences) was a PCR product which comprised the coding region of the NPT gene, FITC-dUTP-labelled according to the manufacturer's instructions with the GenelImages random prime labelling kit (Amersham Biosciences, Freiburg, DE).

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#### 7. Dot Blot Analysis

Genomic DNA was isolated from the cells using a DNA isolation kit according to the manufacturer's instructions (DNA Isolation Kit for Cells and Tissue; Roche 20 Diagnostics GmbH, Mannheim, DE). Various amounts of DNA (10 µg, 5 µg, 2.5 µg, 1.25 µg, 0.63 µg and 0.32 µg) were filtered by the standard method (Ausubel et al., 1994) in an alkaline buffer using a 96 well vacuum manifold (Schleicher & Schüll, Dassel, DE) onto a Hybond N+ nylon membrane (Amersham Biosciences, Freiburg, DE). Untransfected CHO-DG44 cells were used as the negative control. The plasmid pBIN-LC was used as the standard (320 pg, 160 pg, 80 pg, 40 pg, 20 pg, 10 pg, 5 pg, 25 2.5 pg). The probe used for the subsequent non-radioactive hybridisation with the GenelImages CDP-Star Detection Kit (Amersham Biosciences) was a PCR product which comprised the coding region of the NPT gene, FITC-dUTP-labelled according to the manufacturer's instructions with the GenelImages random prime labelling kit (Amersham Biosciences, Freiburg, DE). The chemiluminescence signals were 30 quantified using an ImageMaster VDS-CL (Amersham Biosciences). Then the copy number of the npt genes in the cells in question was determined using the standard series which had been obtained from the signal intensities of the titrated plasmid

DNA. The number of plasmid molecules was calculated using Avogadro's constant and the DNA content of a CHO cell was taken to be about 5 pg.

## 5 Example 1: Mutagenesis of the neomycin-phosphotransferase

The base substitutions in the wild-type NPT-gene needed to prepare the NPT mutants Glu182Gly (SEQ ID NO:3), Trp91Ala (SEQ ID NO:5), Val198Gly (SEQ ID NO:7), Asp227Ala (SEQ ID NO:9), Asp227Val (SEQ ID NO:11), Asp261Gly (SEQ ID NO:13), Asp261Asn (SEQ ID NO:15) and Phe240Ile (SEQ ID NO:17) were carried out by PCR using mutagenic primers (Fig. 3). The vector pBIN (Fig. 1) was used as a template for the PCR mutagenesis. First, the 5' or 3' sections of the mutants were prepared in separate PCR operations. To prepare the mutants Glu182Gly, Trp91Ala and Val198Gly, primer combinations were used for the amplification which consisted of Neofor5 (SEQ ID NO:19) and the relevant mutagenic reverse (rev) primer or Neorev5 (SEQ ID NO:20) and the relevant mutagenic forward (for) primer:

- In the case of NPT mutant Glu182Gly (SEQ ID NO:3) of Neofor5 (SEQ ID NO:19) and E182Grev (SEQ ID NO:24) or of Neorev5 (SEQ ID NO:20) and E182Gfor (SEQ ID NO:23);
- in the case of NPT mutant Trp91Ala (SEQ ID NO:5) of Neofor5 (SEQ ID NO:19) and W91Arev (SEQ ID NO:26) or of Neorev5 (SEQ ID NO:20) and W91Afor (SEQ ID NO:25);
- in the case of NPT mutant Val198Gly (SEQ ID NO:7) of Neofor5 (SEQ ID NO:19) and V198Grev (SEQ ID NO:28) or of Neorev5 (SEQ ID NO:20) and V198Gfor (SEQ ID NO:27).

In order to prepare the mutants Asp227Ala, Asp227Val, Asp261Gly, Asp261Asn and Phe240Ile primer combinations were used for the amplification which consisted of Neofor2 (SEQ ID NO:21) and the relevant mutagenic reverse (rev) primer or of IC49 (SEQ ID NO:22) and the relevant mutagenic forward (for) primer:

- In the case of NPT mutant Asp227Ala (SEQ ID NO:9) of Neofor2 (SEQ ID NO:21) and D227Arev (SEQ ID NO:30) or of IC49 (SEQ ID NO:22) and D227Afor (SEQ ID NO:29);
- in the case of NPT mutant Asp227Val (SEQ ID NO:11) of Neofor2 (SEQ ID NO:21) and D227Vrev (SEQ ID NO:32) or of IC49 (SEQ ID NO:22) and D227Vfor (SEQ ID NO:31);
- in the case of NPT mutant Asp261Gly (SEQ ID NO:13) of Neofor2 (SEQ ID NO:21) and D261Grev (SEQ ID NO:34) or of IC49 (SEQ ID NO:22) and D261Gfor (SEQ ID NO:33);
- in the case of NPT mutant Asp261Asn (SEQ ID NO:15) of Neofor2 (SEQ ID NO:21) and D261Nrev (SEQ ID NO:36) or of IC49 (SEQ ID NO:22) and D261Nfor (SEQ ID NO:35);
- in the case of NPT mutant Phe240Ile (SEQ ID NO:17) of Neofor2 (SEQ ID NO:21) and F240Irev (SEQ ID NO:38) or of IC49 (SEQ ID NO:22) and F240Ifor (SEQ ID NO:37).

Then the coding strand of the 5' section and the complementary strand of the 3' section of the mutants in question were combined by hybridisation in the overlapping region formed by the mutagenic primer sequences, the single strand regions were filled in and the entire product was amplified again in a PCR with the primers Neofor5 (SEQ ID NO:19) and Neorev5 (SEQ ID NO:20) or Neofor2 (SEQ ID NO:21) and IC49 (SEQ ID NO:22). These PCR products were digested with StuI/RsrII (Neofor5/Neorev5 PCR-products) or DraIII/RsrII (Neofor2/IC49 PCR products). Then in the vector pBIN-LC (Fig. 2) part of the wild-type NPT sequence was eliminated by StuI/RsrII digestion or by DraIII/RsrII digestion and replaced by the corresponding fragments of the PCR products. By sequence analysis of both the complementary and the coding strand the desired base substitutions in the various mutants were verified to ensure that the remaining DNA sequence corresponded to the wild-type NPT sequence. In this way the expression vectors pBIN1-LC, pBIN2-LC, pBIN3-LC, pBIN4-LC, pBIN5-LC, pBIN6-LC, pBIN7-LC and pBIN8-LC were generated, which contain the NPT mutants Glu182Gly, Trp91Ala, Val198Gly, Asp227Ala, Asp227Val, Asp261Gly, Asp261Asn or Phe240Ile (Fig. 2).

The mutations inserted in the neomycin phosphotransferase are on the one hand substitutions of more (Val198Gly, Phe240Ile) or less (Trp91Ala, Glu182Gly, Asp227Ala, Asp227Val) conserved amino acids which flank conserved domains, such as e.g. the motifs 1, 2 and 3 (Shaw et al., 1993) (Fig. 4). On the other hand the mutations are located within the conserved motif 3 and relate to a conserved amino acid (Asp261Gly, Asp261Asn).

#### Example 2: Influence of the NPT mutations on the selection of stably transfected mAb-expressing cells

In a co-transfection CHO-DG44 cells were transfected with the plasmid combination pBIDG-HC/pBIN-LC (NPT wild-type), pBIDG-HC/pBIN1-LC (Glu182Gly NPT mutant), pBIDG-HC/pBIN2-LC (Trp91Ala NPT mutant), pBIDG-HC/pBIN3-LC (Val198Gly NPT mutant), pBIDG-HC/pBIN4-LC (Asp227Ala), pBIDG-HC/pBIN5-LC (Asp227Val NPT mutant), pBIDG-HC/pBIN6-LC (Asp261Gly NPT mutant), pBIDG-HC/pBIN7-LC (Asp261Asn NPT mutant) or pBIDG-HC/pBIN8-LC (Phe240Ile NPT mutant) (Fig. 2). In the vector configurations used the two protein chains of a monoclonal humanised IgG2 antibody were each expressed by their own vector, which additionally also codes for a DHFR or neomycin selectable marker in a separate transcription unit. The expression of the product genes is mediated by a CMV-enhancer/hamster ubiquitin/S27a promoter combination. However, comparable data may also be obtained for example with a CMV enhancer/promoter, an SV40- enhancer/hamster ubiquitin /S27a promoter or other promoter combinations.

For each plasmid combination, 5 pools were transfected. In contrast to the cell populations in which selection was done with an NPT wild-type gene, fewer cells survived the initial selection with G418 in the cell populations which had been transfected with a mutated NPT. After a two- to three-week selection of the transfected cell pools in HT-free CHO-S-SFMII medium with the addition of 400 µg/mL of G418 the antibody titre in the cell culture supernatants was determined by ELISA over six runs. Fig. 5 shows the averages of the titres and productivities determined from the pools in the test. Compared with the use of an NPT wild-type

gene as the selectable marker, all the cell pools which had been selected with an NPT mutant showed on average an increase in productivity and titre by a factor of 1.4 - 14.6 and 1.4 - 10.8, respectively (Fig. 5). The best selective enrichment of cells with a higher basic productivity could thus be obtained with the NPT mutants

5 Asp227Val and Asp261Gly, with increases in average productivity by a factor of 14.6 and 9.3, respectively.

The vector pBIDG-HC contains another selectable marker, GFP. The GFP is transcriptionally linked to the heavy chain via an IRES element. The resulting

10 correlation between the expression of the target protein and the selectable marker GFP therefore also makes it possible to rapidly evaluate the level and distribution of the expression levels in the transfected cell populations on the basis of the GFP fluorescence determined in FACS analyses. After two to three weeks' selection of the transfected cell pools in HT-free CHO-S-SFMII medium with the addition of G418

15 the GFP fluorescence was measured in a FACS analysis (Fig. 6). The GFP fluorescence signals in fact correlated with the titre data obtained for the monoclonal IgG2 antibody. Pools selected with the NPT mutants Asp227Val, Asp261Gly, Asp161Asn and Phe240Ile also had the higher proportion of cells with a high GFP fluorescence, followed by the cells selected with the NPT mutants Trp91Ala,

20 Asp227Ala, Glu182Gly and Val198Gly.

### **Example 3: Determining and comparing the NPT enzyme activity**

25 In order to compare the enzyme activity of the NPT mutants with that of the NPT wild-type a dot assay was carried out to determine the NPT activity in cell extracts, based on the procedure of Platt et al. 1987. Cell extracts were prepared from two different mAb-expressing cell pools which had been transfected and selected either with the NPT wild-type gene (SEQ ID NO:1) or with the NPT mutants Glu182Gly (SEQ ID

30 NO:3), Trp91Ala (SEQ ID NO:5), Val198Gly (SEQ ID NO:7), Asp227Ala (SEQ ID NO:9), Asp227Val (SEQ ID NO:11), Asp261Gly (SEQ ID NO:13), Asp261Asn (SEQ ID NO:15) or Phe240Ile (SEQ ID NO:17). Cell extracts from untransfected CHO-DG44 cells were used as the negative control.

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## PATENT CLAIMS

- 5 1. Modified neomycin phosphotransferase gene *characterised in that* the modified neomycin phosphotransferase gene at amino acid position 91, 198 and/or 240 in relation to the wild-type gene codes for a different amino acid than the wild-type neomycin phosphotransferase gene.
- 10 2. Modified neomycin phosphotransferase gene according to claim 1, *characterised in that* the modified neomycin phosphotransferase which is encoded by the neomycin phosphotransferase gene has a lower enzyme activity than the wild-type neomycin phosphotransferase.
- 15 3. Modified neomycin phosphotransferase gene according to claim 1 or 2 *characterised in that* the modified neomycin phosphotransferase gene compared with the wild-type gene codes for alanine at amino acid position 91 in relation to the wild-type gene, for glycine at amino acid position 198, and/or for isoleucine at amino acid position 240.
- 20 4. Modified neomycin phosphotransferase gene according to one of claims 1 to 3 *characterised in that* the modified neomycin phosphotransferase gene codes for an amino acid sequence according to SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:18.
- 25 5. Modified neomycin phosphotransferase gene according to one of claims 1 to 4 containing or consisting of a sequence according to SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:17.
- 30 6. Modified neomycin phosphotransferase gene *characterised in that* the modified neomycin phosphotransferase gene compared with the wild-type gene codes for glycine at amino acid position 182 in relation to the wild-type gene, for alanine or valine at amino acid position 227 and/or for glycine at amino acid position 261.

7. Modified neomycin phosphotransferase gene according to claim 6 *characterised in that* the modified neomycin phosphotransferase gene codes for an amino acid sequence according to SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:12 or SEQ ID NO:14.

5

8. Modified neomycin phosphotransferase gene according to one of claims 6 or 7 containing or consisting of a sequence according to SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:11 or SEQ ID NO:13.

10 9. Modified neomycin phosphotransferase encoded by a modified neomycin phosphotransferase gene according to one of claims 1 to 8.

10. Eukaryotic expression vector containing a modified neomycin phosphotransferase gene according to one of claims 1 to 8.

15

11. Expression vector according to claim 10, containing a multiple cloning site for the incorporation of a gene which codes for a protein/product of interest and is functionally linked to a heterologous promoter.

20 12. Expression vector according to claim 10 containing a heterologous gene of interest functionally linked to a heterologous promoter.

13. Expression vector according to claim 12 *characterised in that* it contains one or more enhancers functionally linked to the promoter or promoters.

25

14. Expression vector according to claim 13 *characterised in that* the enhancer is a CMV or SV40 enhancer.

30

15. Expression vector according to one of claims 11 to 14 *characterised in that* it contains a hamster ubiquitin/S27a promoter.

16. Expression vector according to claim 15 *characterised in that* the heterologous gene of interest is under the control of the ubiquitin/S27a promoter.

17. Expression vector according to one of claims 12 to 16 *characterised in that* it additionally contains a gene for a fluorescent protein which is/will optionally be functionally linked to the gene of interest and the heterologous promoter.

5

18. Expression vector according to claim 17, characterised in that it additionally contains an internal ribosome entry site (IRES) which enables bicistronic expression of the gene which codes for a fluorescent protein, and of the gene which codes for a protein/product of interest, under the control of a heterologous promoter.

10

19. Expression vector according to claim 17 or 18, characterised in that the gene which codes for a fluorescent protein, and the modified neomycin-phosphotransferase gene are located in one or in two separate transcription units.

15

20. Mammalian cell containing a modified neomycin phosphotransferase gene according to one of claims 1 to 8.

21. Mammalian cell which has been transfected with an expression vector according to one of Claims 10 to 16.

20

22. Mammalian cell which has been transfected with an expression vector according to one of Claims 17 to 19.

23. Mammalian cell according to one of claims 20 to 22, characterised in that it has additionally been transfected with a gene for an amplifiable selectable marker.

25

24. Mammalian cell according to claim 23, characterised in that the amplifiable selectable marker gene is dihydrofolate-reductase (DHFR).

30

25. Mammalian cell according to one of claims 20 to 24, characterised in that the mammalian cell is a rodent cell.

26. Mammalian cell according to claim 25, characterised in that the rodent cell is a CHO or BHK cell.

5 27. Method of enriching mammalian cells, characterised in that

- (i) a pool of mammalian cells is transfected with a gene for a modified neomycin-phosphotransferase according to one of claims 1 to 8 or with a D261N neomycin phosphotransferase mutant;
- (ii) the mammalian cells are cultivated under conditions which allow  
10 expression of the modified neomycin-phosphotransferase gene; and
- (iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene.

15

28. Method of obtaining and selecting mammalian cells which express at least one heterologous gene of interest, characterised in that

- (i) a pool of mammalian cells is transfected with at least one gene of interest and a gene for a modified neomycin-phosphotransferase according to one of claims 1  
20 to 8 or with a D261N neomycin phosphotransferase mutant;
- (ii) the mammalian cells are cultivated under conditions which allow expression of the gene or genes of interest and of the modified neomycin-phosphotransferase gene; and
- (iii) the mammalian cells are cultivated in the presence of at least one selecting  
25 agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene.

30

29. Method according to claim 28, characterised in that the mammalian cells are additionally transfected with a gene for an amplifiable selectable marker and the selected mammalian cells are subjected to at least one gene amplification step, the amplifiable selectable marker gene preferably being DHFR and the gene amplification preferably being carried out by the addition of methotrexate.

30. Method of obtaining and selecting mammalian cells which express at least one heterologous gene of interest, characterised in that

(i) recombinant mammalian cells are transformed with an expression vector

according to one of Claims 17 to 19;

(ii) are cultivated under conditions which allow expression of the gene (or genes) of interest, of the gene which codes for a fluorescent protein, and of the modified neomycin-phosphotransferase gene;

(iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene; and

(iv) the mammalian cells are sorted by flow-cytometric analysis.

31. Method according to claim 30, characterised in that the mammalian cells are additionally transfected with a gene for an amplifiable selectable marker and the cells sorted by flow-cytometric analysis are subjected to at least one gene amplification step, the amplifiable selectable marker gene preferably being DHFR and the gene amplification preferably being carried out by the addition of methotrexate.

32. Method of producing at least one protein of interest in recombinant mammalian cells, characterised in that

(i) a pool of mammalian cells is transfected with at least one gene of interest and one gene for a modified neomycin-phosphotransferase according to one of claims 1 to 8 or with a D261N neomycin phosphotransferase mutant;

(ii) the cells are cultivated under conditions which allow expression of the gene (or genes) of interest and of the modified neomycin-phosphotransferase;

(iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene; and

(iv) the protein(s) of interest is or are obtained from the mammalian cells or the culture supernatant.

33. Method of producing at least one protein of interest in recombinant mammalian cells, characterised in that

(i) recombinant mammalian cells are transformed with an expression vector according to one of Claims 17 to 19;

(ii) are cultivated under conditions which allow expression of the gene (or genes) of interest, of the gene which codes for a fluorescent protein, and of the modified neomycin-phosphotransferase gene;

(iii) the mammalian cells are cultivated in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene; and

(iv) the mammalian cells are sorted by flow-cytometric analysis.

34. Method of producing at least one protein of interest, characterised in that

(i) mammalian cells according to one of Claims 23 or 24 are cultivated under conditions which allow expression of the gene of interest, of the modified neomycin-phosphotransferase gene and of the amplifiable selectable marker gene;

(ii) the mammalian cells are cultivated and selected in the presence of at least one selecting agent which acts selectively on the growth of mammalian cells, and gives preference to the growth of those cells which express the modified neomycin-phosphotransferase gene;

(iii) the selected mammalian cells are subjected to at least one gene amplification step; and

(iv) the protein(s) of interest is or are subsequently obtained from the mammalian cells or the culture supernatant.

35. Method according to one of Claims 32 to 34, characterised in that the mammalian cells are transfected with at least two genes of interest which code for a heteromeric protein/product, and

- (i) are cultivated under conditions which allow expression of the subunits of the heteromeric protein/product; and
- (ii) the heteromeric protein/product is isolated from the culture or culture medium.

5 36. Method according to one of Claims 32 to 35, characterised in that the sorted mammalian cells exhibit an average specific productivity of more than 5pg of the desired gene product (or products) per day and per cell.

10 37. Method according to claim 32 or 35, characterised in that the sorted host cells exhibit an average specific productivity of more than 20pg of the desired gene product (or products) per day and per cell.

38. Method according to one of Claims 32 to 36, characterised in that the mammalian cell is a rodent cell.

15

39. Method according to Claim 38, characterised in that the rodent cell is a CHO or BHK cell.

20

40. Method according to one of Claims 32 to 39, characterised in that the mammalian cells are cultivated in suspension culture.

41. Method according to one of Claims 32 to 40, characterised in that the mammalian cells are cultivated serum-free.

25

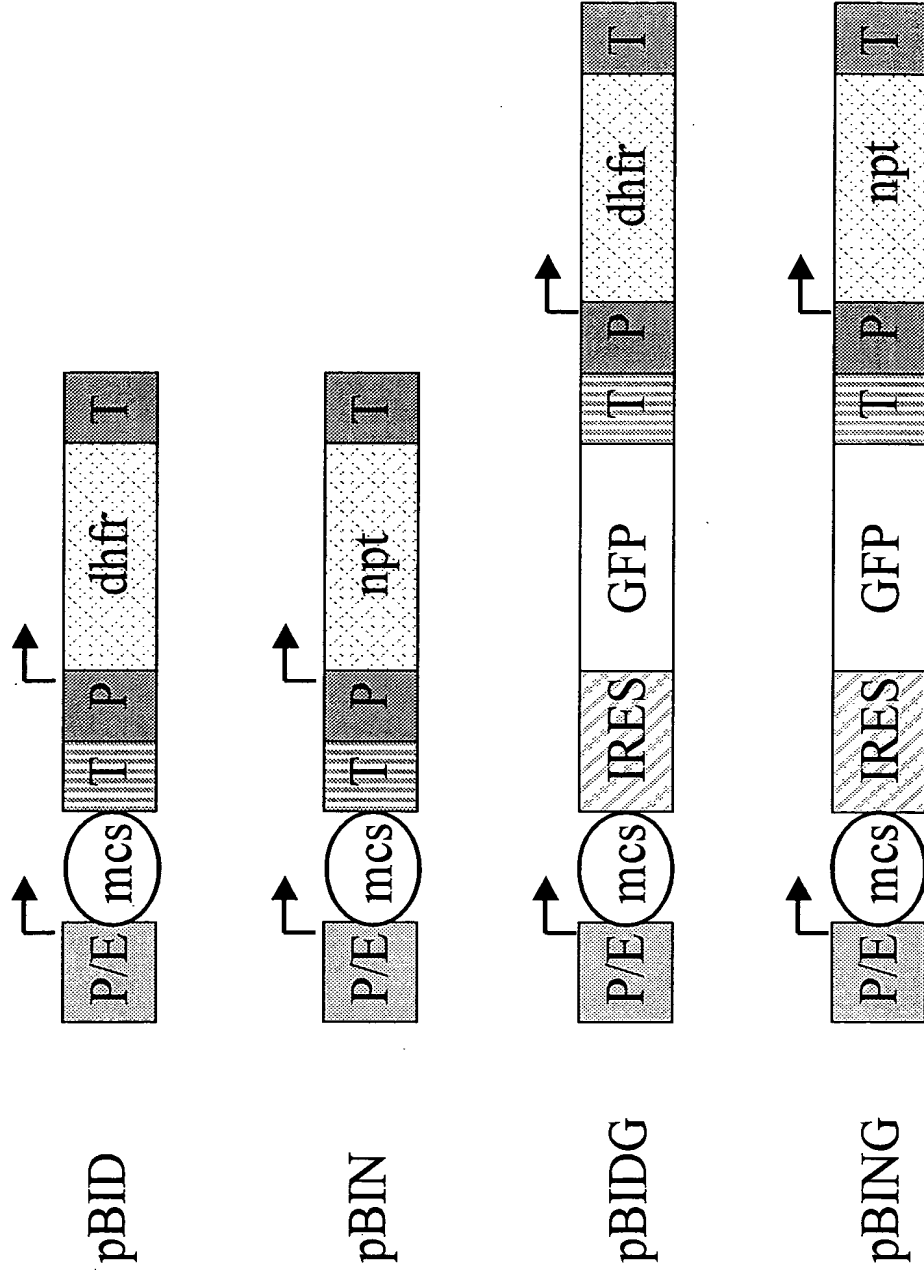
## ABSTRACT

5     NEW NEOMYCIN-PHOSPHOTRANSFERASE GENES AND METHODS FOR THE  
      SELECTION OF RECOMBINANT CELLS PRODUCING HIGH LEVELS OF A  
          DESIRED GENE PRODUCT

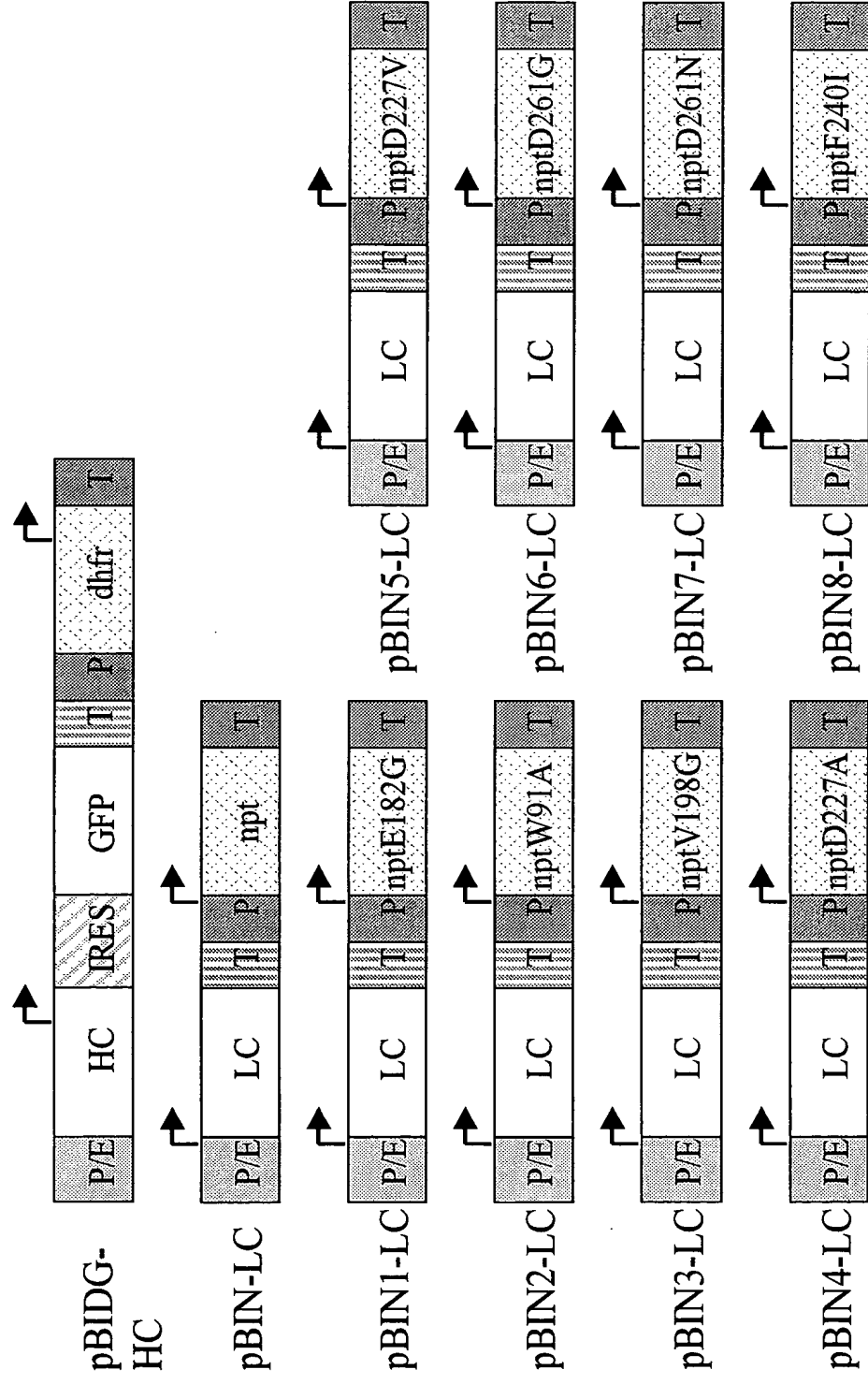
10    The invention relates to new modified neomycin phosphotransferase genes  
      and their use in a selection method for high-producing recombinant cells. The  
      invention further relates to expression vectors which contain a modified  
      neomycin phosphotransferase gene and a gene of interest functionally linked  
      to a heterologous promoter and a method of preparing heterologous gene  
      products using these expression vectors.



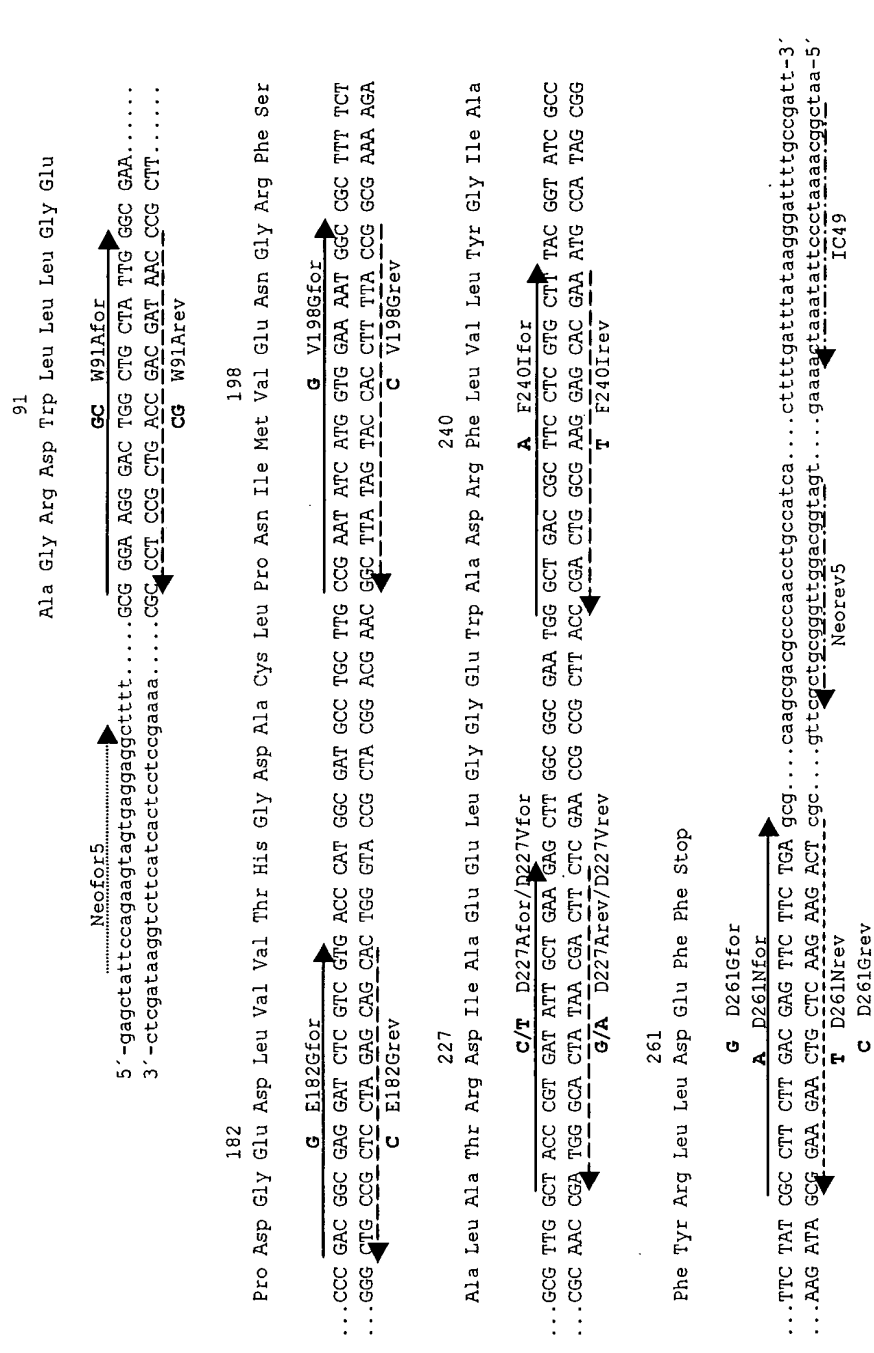
**Figure 1**



**Figure 2**



# Figure 3



Glu182Gly: GAG => GGT; Trp91Ala : TGG => GCG; Val198Gly: GTG => GGG; Asp227Val: GAT => GTT  
Asp227Ala: GAT => GCT; Asp261Gly: GAC => GGC; Asp261Asn: GAC => AAC; Phe240Ile: TTC => ATC

**Figure 4**

1 MIEQDGLHAG SPAAWVERLF GYDWAQQTIG CSDAAVERLS AQGRPVLFVK  
91  
51 TDLSGALNEL QDEAARLSWL ATTGVPCAAY LDVVTEAGRD ★ WLLGEVPGQ  
101 DLLSSHLAPA EKVSIMADAM RRLHTLDPAT CPE<sup>182</sup>DHQAKHR IERARTRMEA  
151 GLVDQDDLDE EHQGLAPAE<sup>189</sup>L FARLKASMPD GEDLVVTHGD ★ AC<sup>189</sup>LPNIMVEN  
227 240 motif 1  
201 GRFSGFIDCG RLG<sup>227</sup>VADRYQD IALATRDIAE ★ ELGGEWADRF LVL<sup>240</sup>YGIAAPD  
motif 2<sub>261</sub>  
251 SQRIAFYRLL DEFF ★  
motif 3

Figure 5

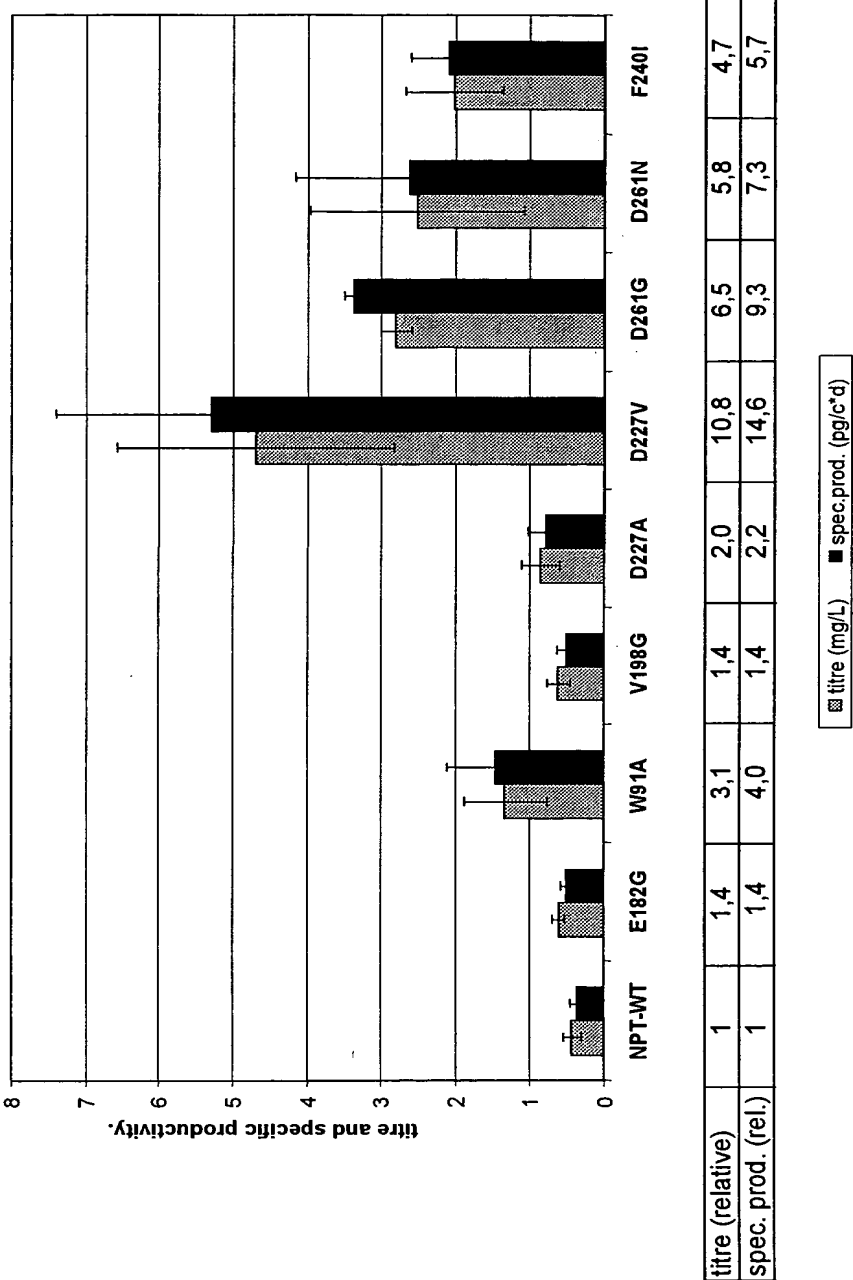
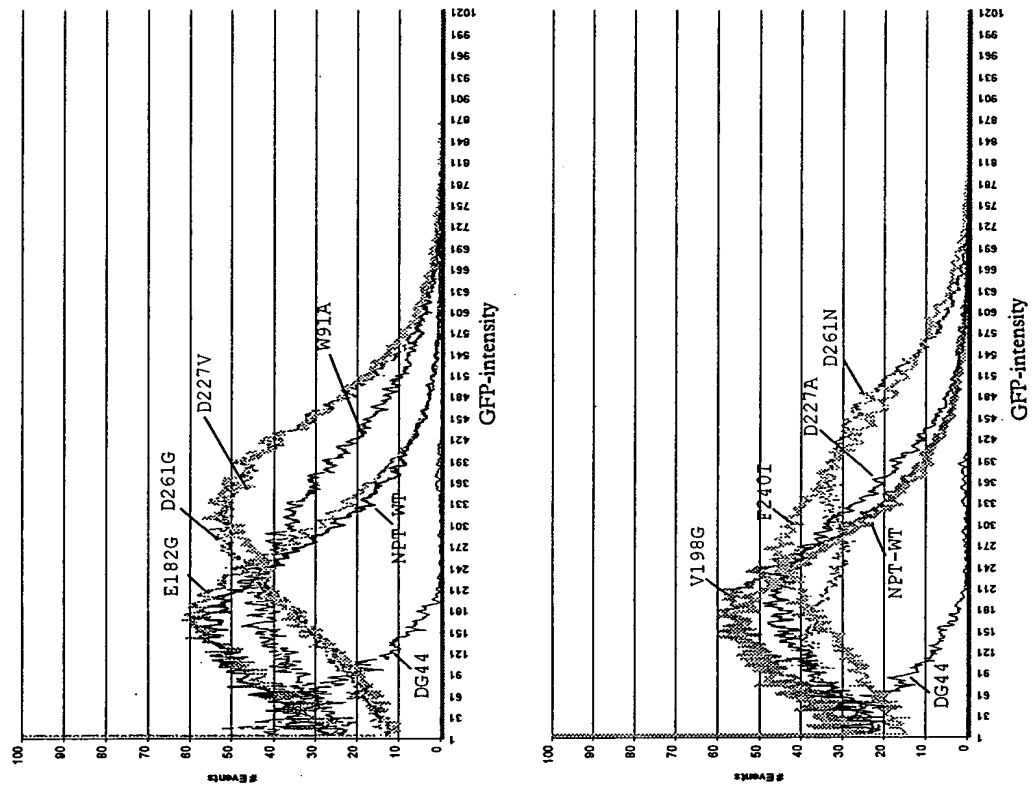


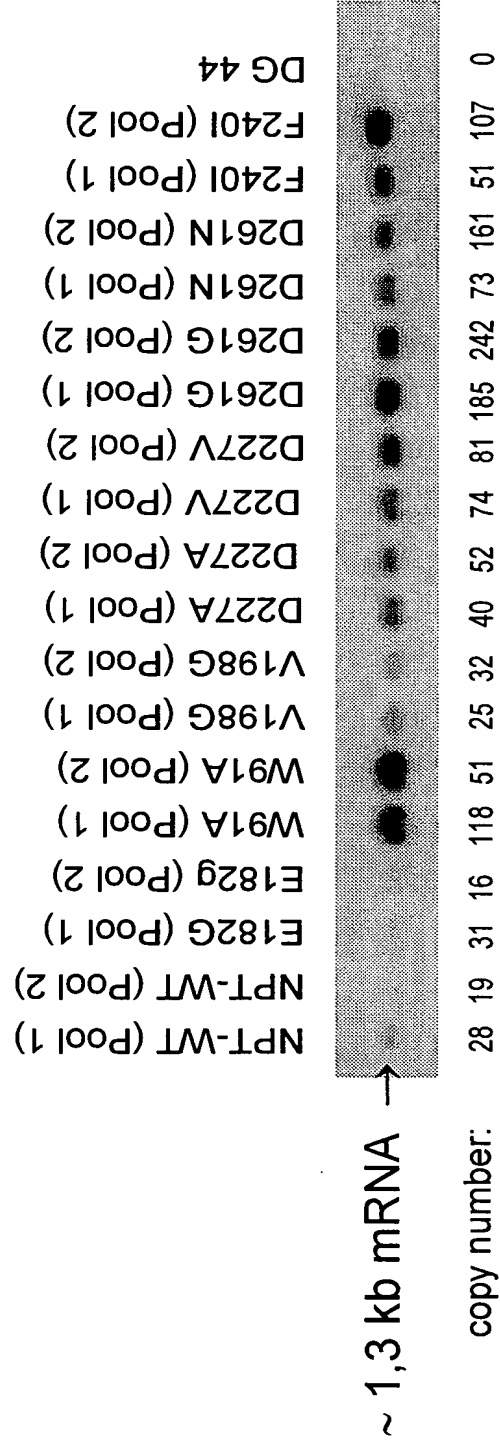
Figure 6



**Figure 7**

<b>NPT-variant</b>	<b>% enzyme activity (WT-NPT = 100%)</b>
NPT-wild-type	100,0
Glu182Gly	25,8
Trp91Ala	53,2
Val198Gly	61,9
Asp227Ala	26,9
Asp227Val	16,4
Asp261Gly	3,1
Asp261Asn	1,5
Phe240Ile	23,4

**Figure 8**



**BEST AVAILABLE COPY**



## SEQUENCE LISTING

&lt;110&gt; BOEHRINGER INGELHEIM PHARMA GMBH &amp; CO KG

5 <120> NEW NEOMYCIN-PHOSPHOTRANSFERASE GENES AND METHODS FOR THE  
SELECTION OF RECOMBINANY CELLS PRODUCING HIGH LEVELS OF A DESIRED GENE  
PRODUCT

&lt;130&gt; Case 1-1503

10

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 39

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&lt;170&gt; PatentIn Ver. 2.1

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&lt;213&gt; Escherichia coli

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Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
35 40 45

Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala  
 50 55 60

5 Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
 65 70 75 80

Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu  
 85 90 95

10 Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
 100 105 110

Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
 115 120 125

15 Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala  
 130 135 140

Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu  
 145 150 155 160

Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala  
 165 170 175

25 Ser Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys  
 180 185 190

Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp  
 195 200 205

30 Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala  
 210 215 220

Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe  
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 30 Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
 35 40 45  
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 50 55 60  
 35 Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
 65 70 75 80  
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 85 90 95  
 40 Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
 100 105 110  
 45 Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
 115 120 125  
 Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala  
 130 135 140  
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5 Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala  
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Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe  
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30 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
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	Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe		
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5	Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala		
	50	55	60
10	Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val		
	65	70	75
	Leu Asp Val Val Thr Glu Ala Gly Arg Asp Ala Leu Leu Leu Gly Glu		
	85	90	95
15	Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys		
	100	105	110
	Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro		
	115	120	125
20	Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala		
	130	135	140
25	Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu		
	145	150	155
	Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala		
	165	170	175
30	Ser Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys		
	180	185	190
	Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp		
	195	200	205
35	Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala		
	210	215	220
40	Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe		
	225	230	235
	Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe		
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Neomycin mutant V198G

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 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
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 Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser  
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 35 Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
 35 40 45  
 Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala  
 50 55 60  
 40 Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
 65 70 75 80  
 Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu  
 85 90 95  
 45 Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
 100 105 110  
 Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
 115 120 125  
 50 Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala  
 130 135 140  
 55 Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu  
 145 150 155 160

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							165										175
5	Ser	Met	Pro	Asp	Gly	Glu	Asp	Leu	Val	Val	Thr	His	Gly	Asp	Ala	Cys	
				180					185					190			
	Leu	Pro	Asn	Ile	Met	Gly	Glu	Asn	Gly	Arg	Phe	Ser	Gly	Phe	Ile	Asp	
			195					200					205				
10	Cys	Gly	Arg	Leu	Gly	Val	Ala	Asp	Arg	Tyr	Gln	Asp	Ile	Ala	Leu	Ala	
		210					215					220					
	Thr	Arg	Asp	Ile	Ala	Glu	Glu	Leu	Gly	Gly	Glu	Trp	Ala	Asp	Arg	Phe	
	225					230					235					240	
15	Leu	Val	Leu	Tyr	Gly	Ile	Ala	Ala	Pro	Asp	Ser	Gln	Arg	Ile	Ala	Phe	
				245						250					255		
	Tyr	Arg	Leu	Leu	Asp	Glu	Phe	Phe									
20				260													
	<210> 9																
	<211> 795																
25	<212> DNA																
	<213> Artificial sequence...																
	<220>																
	<223> Description of the artificial sequence:																
30	Neomycin mutant D227A																
	<400> 9																
	atgattgaac	aagatggatt	gcacgcaggt	tctccggccg	cttgggtgga	gaggctattc	60										
	ggctatgact	gggcacaaca	gacaatcggc	tgctctgatg	ccgccgtggt	ccggctgtca	120										
35	gcgcaggggc	gcccggttct	ttttgtcaag	accgacctgt	ccggtgccct	gaatgaactg	180										
	caagacgagg	cagcgcggtc	atcgtggctg	gccacgacgg	gcgttccttg	cgcagctgtg	240										
	ctcgacgttg	tactgaagc	gggaaggac	tggtctgctat	tgggcgaagt	gccggggcag	300										
	gatctcctgt	catctcacct	tgctcctgcc	gagaaagtat	ccatcatggc	tgatgcaatg	360										
	cggcggtctg	atagccttga	tccggctacc	tgcccattcg	accaccaagc	gaaacatcgc	420										
40	atcgagcgag	cacgtactcg	gatggaagcc	ggtcttgtcg	atcaggatga	tctggacgaa	480										
	gagcatcagg	ggctcgcgcc	agccgaactg	ttcgccaggc	tcaaggcgag	catgcccgac	540										
	ggcgaggatc	tcgtcgtgac	ccatggcgat	gcctgcttgc	cgaatatcat	ggtggaaaat	600										
	ggccgctttt	ctggattcat	cgactgtggc	cggctgggtg	tggcggaccg	ctatcaggac	660										
	atagcgttgg	ctaccctgtc	tattgctgaa	gagcttggcg	gcgaatgg												

```

50      <210> 10
        <211> 264
        <212> PRT
        <213> Artificial sequence

```

55 <223> Description of the artificial sequence:  
Neomycin mutant D227A

&lt;400&gt; 10

	Met	Ile	Glu	Gln	Asp	Gly	Leu	His	Ala	Gly	Ser	Pro	Ala	Ala	Trp	Val	
	1				5					10					15		
5	Glu	Arg	Leu	Phe	Gly	Tyr	Asp	Trp	Ala	Gln	Gln	Thr	Ile	Gly	Cys	Ser	
				20					25					30			
	Asp	Ala	Ala	Val	Phe	Arg	Leu	Ser	Ala	Gln	Gly	Arg	Pro	Val	Leu	Phe	
				35				40					45				
10	Val	Lys	Thr	Asp	Leu	Ser	Gly	Ala	Leu	Asn	Glu	Leu	Gln	Asp	Glu	Ala	
		50					55					60					
	Ala	Arg	Leu	Ser	Trp	Leu	Ala	Thr	Thr	Gly	Val	Pro	Cys	Ala	Ala	Val	
15		65				70					75					80	
	Leu	Asp	Val	Val	Thr	Glu	Ala	Gly	Arg	Asp	Trp	Leu	Leu	Leu	Gly	Glu	
					85					90					95		
20	Val	Pro	Gly	Gln	Asp	Leu	Leu	Ser	Ser	His	Leu	Ala	Pro	Ala	Glu	Lys	
				100					105					110			
	Val	Ser	Ile	Met	Ala	Asp	Ala	Met	Arg	Arg	Leu	His	Thr	Leu	Asp	Pro	
			115					120					125				
25	Ala	Thr	Cys	Pro	Phe	Asp	His	Gln	Ala	Lys	His	Arg	Ile	Glu	Arg	Ala	
		130					135					140					
	Arg	Thr	Arg	Met	Glu	Ala	Gly	Leu	Val	Asp	Gln	Asp	Asp	Leu	Asp	Glu	
30		145				150					155					160	
	Glu	His	Gln	Gly	Leu	Ala	Pro	Ala	Glu	Leu	Phe	Ala	Arg	Leu	Lys	Ala	
					165					170					175		
35	Ser	Met	Pro	Asp	Gly	Glu	Asp	Leu	Val	Val	Thr	His	Gly	Asp	Ala	Cys	
				180					185					190			
	Leu	Pro	Asn	Ile	Met	Val	Glu	Asn	Gly	Arg	Phe	Ser	Gly	Phe	Ile	Asp	
			195					200					205				
40	Cys	Gly	Arg	Leu	Gly	Val	Ala	Asp	Arg	Tyr	Gln	Asp	Ile	Ala	Leu	Ala	
		210					215					220					
	Thr	Arg	Ala	Ile	Ala	Glu	Glu	Leu	Gly	Gly	Glu	Trp	Ala	Asp	Arg	Phe	
45		225				230					235					240	
	Leu	Val	Leu	Tyr	Gly	Ile	Ala	Ala	Pro	Asp	Ser	Gln	Arg	Ile	Ala	Phe	
					245					250					255		
50	Tyr	Arg	Leu	Leu	Asp	Glu	Phe	Phe									
					260												

&lt;210&gt; 11

55 &lt;211&gt; 795

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence



&lt;220&gt;

&lt;223&gt; Description of the artificial sequence:

Neomycin mutant D227V

5

&lt;400&gt; 11

atgattgaac aagatggatt gcacgcaggt tctccggccg cttgggtgga gaggctattc 60  
 ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtgtt ccggctgtca 120  
 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
 10 caagacgagg cagcgcggct atcgtggctg gccacgacgg gcgttccttg cgcagctgtg 240  
 ctcgacgttg tactgaagc gggaaggac tggctgctat tgggcgaagt gccggggcag 300  
 gatctcctgt catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 360  
 cggcggctgc atacgcttga tccggctacc tgcccattcg accaccaagc gaaacatcgc 420  
 atcgagcgag cactactcg gatggaagcc ggtcttgctg atcaggatga tctggacgaa 480  
 15 gagcatcagg ggctcgcgcc agccgaactg ttccgccaggc tcaaggcgag catgcccgcac 540  
 ggcgaggatc tcgtcgtgac ccatggcgat gcctgcttgc cgaatatcat ggtggaaaat 600  
 ggccgctttt ctggattcat cgactgtggc cggctgggtg tggcggaccg ctatcaggac 660  
 atagcgttgg ctaccctgtg tattgtgaa gagcttggcg gcgaatgggc tgaccgcttc 720  
 ctcgtgcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt 780  
 20 gacgatttct tctga 795

&lt;210&gt; 12

&lt;211&gt; 264

25 &lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Description of the artificial sequence:

30 Neomycin mutant D227V

&lt;400&gt; 12

Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val  
 1 5 10 15  
 35 Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser  
 20 25 30  
 40 Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
 35 40 45  
 Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala  
 50 55 60  
 45 Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
 65 70 75 80  
 Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu  
 85 90 95  
 50 Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
 100 105 110  
 55 Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
 115 120 125  
 Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala

130 135 140

Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu  
145 150 155 160

5 Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala  
165 170 175

10 Ser Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys  
180 185 190

Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp  
195 200 205

15 Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala  
210 215 220

Thr Arg Val Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe  
225 230 235 240

20 Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe  
245 250 255

Tyr Arg Leu Leu Asp Glu Phe Phe  
25 260

<210> 13  
<211> 795  
30 <212> DNA  
<213> Artificial sequence

<220>  
<223> Description of the artificial sequence:  
35 Neomycin mutant D261G

<400> 13  
atgattgaac aagatggatt gcacgcaggt tctccggccg cttgggtgga gaggctattc 60  
ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtgtt ccggctgtca 120  
40 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
caagacgagg cagcgcggct atcgtggctg gccacgacgg gcgttccttg cgcagctgtg 240  
ctcgacgttg tactgaagc gggaaggac tggctgctat tgggcgaagt gccggggcag 300  
gatctcctgt catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 360  
cggcggctgc atacgcttga tccggctacc tgcccattcg accaccaagc gaaacatcgc 420  
45 atcgagcgag cacgtactcg gatggaagcc ggtcttgtcg atcaggatga tctggacgaa 480  
gagcatcagg ggctcgcgcc agccgaactg ttccgccaggc tcaaggcgag catgcccgac 540  
ggcgaggatc tcgtcgtgac ccatggcgat gcctgcttgc cgaatatcat ggtggaaaat 600  
ggccgctttt ctggattcat cgactgtggc cggttgggtg tggcggaccg ctatcaggac 660  
atagcgttgg ctaccctgta tattgctgaa gagcttggcg gcgaatgggc tgaccgcttc 720  
50 ctcggtgcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt 780  
ggcgagttct tctga 795

<210> 14  
55 <211> 264  
<212> PRT  
<213> Artificial sequence

<223> Description of the artificial sequence:  
Neomycin mutant D261G

<400> 14

Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val  
1 5 10 15

Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser  
20 25 30

Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
35 40 45

Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala  
50 55 60

Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
65 70 75 80

Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu  
85 90 95

Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
100 105 110

Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
115 120 125

Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala  
130 135 140

Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu  
145 150 155 160

Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala  
165 170 175

Ser Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys  
180 185 190

Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp  
195 200 205

Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala  
210 215 220

Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe  
225 230 235 240

Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe  
245 250 255

Tyr Arg Leu Leu Gly Glu Phe Phe  
260

&lt;210&gt; 15

&lt;211&gt; 795

&lt;212&gt; DNA

5 &lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Description of the artificial sequence:

Neomycin mutant D261N

10

&lt;400&gt; 15

atgattgaac aagatggatt gcacgcaggt tctccggccg cttgggtgga gaggtatttc 60  
 ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtgtt ccggctgtca 120  
 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
 15 caagacgagg cagcgcggct atcgtggctg gccacgacgg gcgttccttg cgcagctgtg 240  
 ctcgacgttg tcaactgaagc gggaaggac tggctgctat tgggcgaagt gccggggcag 300  
 gatctcctgt catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 360  
 cggcggtgc atacgcttga tccggctacc tgcccattcg accaccaagc gaaacatcgc 420  
 atcgagcgag cacgtactcg gatggaagcc ggtcttgtcg atcaggatga tctggacgaa 480  
 20 gagcatcagg ggctcgcgcc agccgaactg ttgccaggc tcaaggcgag catgcccagc 540  
 ggcgaggatc tcgtcgtgac ccatggcgat gcctgcttgc cgaatatcat ggtggaaaat 600  
 ggccgctttt ctggattcat cgactgtggc cggctgggtg tggcggaccg ctatcaggac 660  
 atagcgttgg ctaccctgga tattgctgaa gagcttggcg gcgaatgggc tgaccgcttc 720  
 ctcgtgcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt 780  
 25 aacgagttct tctga 795

&lt;210&gt; 16

&lt;211&gt; 264

30 &lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Description of the artificial sequence:

35 Neomycin mutant D261N

&lt;400&gt; 16

Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val  
 1 5 10 15  
 40 Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser  
 20 25 30  
 45 Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe  
 35 40 45  
 Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala  
 50 55 60  
 50 Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val  
 65 70 75 80  
 Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu  
 85 90 95  
 55 Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys  
 100 105 110

Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro  
115 120 125

5 Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala  
130 135 140

Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu  
145 150 155 160

10 Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala  
165 170 175

15 Ser Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys  
180 185 190

Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp  
195 200 205

20 Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala  
210 215 220

Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe  
225 230 235 240

25 Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe  
245 250 255

30 Tyr Arg Leu Leu Asn Glu Phe Phe  
260

<210> 17  
<211> 795  
35 <212> DNA  
<213> Artificial sequence

<220>  
<223> Description of the artificial sequence:  
40 Neomycin mutant F240I

<400> 17  
atgattgaac aagatggatt gcacgcaggt tctccggccg cttgggtgga gaggctattc 60  
ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtggt ccggctgtca 120  
45 gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180  
caagacgagg cagcgcggtc atcgtggctg gccacgacgg gcgttccttg cgcagctgtg 240  
ctcgacgttg tcaactgaagc gggaaggagc tggctgctat tgggcgaagt gccggggcag 300  
gatctcctgt catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 360  
cggcggtctgc atacgcttga tccggctacc tgcccattcg accaccaagc gaaacatcgc 420  
50 atcgagcgag cacgtactcg gatggaagcc ggtcttgtcg atcaggatga tctggacgaa 480  
gagcatcagg ggctcgcgcc agccgaactg ttcgccaggc tcaaggcgag catgcccgcac 540  
ggcgaggatc tcgtcgtgac ccatggcgat gcctgcttgc cgaatatcat ggtggaaaat 600  
ggccgctttt ctggattcat cgactgtggc cggtctgggt tggcggaccg ctatcaggac 660  
atagcgtttg ctaccctgta tattgctgaa gagcttggcg gcgaatgggc tgaccgcac 720  
55 ctctgtcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt 780  
gacgagttct tctga 795

&lt;210&gt; 18

&lt;211&gt; 264

&lt;212&gt; PRT

5 &lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Description of the artificial sequence:

Neomycin mutant F240I

10

&lt;400&gt; 18

Met	Ile	Glu	Gln	Asp	Gly	Leu	His	Ala	Gly	Ser	Pro	Ala	Ala	Trp	Val
1				5					10					15	

15	Glu	Arg	Leu	Phe	Gly	Tyr	Asp	Trp	Ala	Gln	Gln	Thr	Ile	Gly	Cys	Ser
			20						25					30		

	Asp	Ala	Ala	Val	Phe	Arg	Leu	Ser	Ala	Gln	Gly	Arg	Pro	Val	Leu	Phe
			35					40					45			

20	Val	Lys	Thr	Asp	Leu	Ser	Gly	Ala	Leu	Asn	Glu	Leu	Gln	Asp	Glu	Ala
		50					55					60				

25	Ala	Arg	Leu	Ser	Trp	Leu	Ala	Thr	Thr	Gly	Val	Pro	Cys	Ala	Ala	Val
	65					70					75					80

	Leu	Asp	Val	Val	Thr	Glu	Ala	Gly	Arg	Asp	Trp	Leu	Leu	Leu	Gly	Glu
					85					90					95	

30	Val	Pro	Gly	Gln	Asp	Leu	Leu	Ser	Ser	His	Leu	Ala	Pro	Ala	Glu	Lys
				100					105					110		

	Val	Ser	Ile	Met	Ala	Asp	Ala	Met	Arg	Arg	Leu	His	Thr	Leu	Asp	Pro
			115					120					125			

35	Ala	Thr	Cys	Pro	Phe	Asp	His	Gln	Ala	Lys	His	Arg	Ile	Glu	Arg	Ala
		130					135					140				

40	Arg	Thr	Arg	Met	Glu	Ala	Gly	Leu	Val	Asp	Gln	Asp	Asp	Leu	Asp	Glu
	145					150					155					160

	Glu	His	Gln	Gly	Leu	Ala	Pro	Ala	Glu	Leu	Phe	Ala	Arg	Leu	Lys	Ala
					165				170						175	

45	Ser	Met	Pro	Asp	Gly	Glu	Asp	Leu	Val	Val	Thr	His	Gly	Asp	Ala	Cys
				180					185					190		

	Leu	Pro	Asn	Ile	Met	Val	Glu	Asn	Gly	Arg	Phe	Ser	Gly	Phe	Ile	Asp
			195					200					205			

50	Cys	Gly	Arg	Leu	Gly	Val	Ala	Asp	Arg	Tyr	Gln	Asp	Ile	Ala	Leu	Ala
		210					215					220				

55	Thr	Arg	Asp	Ile	Ala	Glu	Glu	Leu	Gly	Gly	Glu	Trp	Ala	Asp	Arg	Ile
	225					230					235					240

	Leu	Val	Leu	Tyr	Gly	Ile	Ala	Ala	Pro	Asp	Ser	Gln	Arg	Ile	Ala	Phe
--	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

245

250

255

Tyr Arg Leu Leu Asp Glu Phe Phe  
260

5

&lt;210&gt; 19

&lt;211&gt; 21

&lt;212&gt; DNA

10 &lt;213&gt; Artificial sequence

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide Neofor5

15

&lt;400&gt; 19

ttccagaagt agtgaggagg c

21

20

&lt;210&gt; 20

&lt;211&gt; 19

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

25

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide Neorev5

30

&lt;400&gt; 20

atggcaggtt gggcgtcgc

19

35

&lt;210&gt; 21

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

40

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide Neofor2

&lt;400&gt; 21

gaactgttcg ccaggctcaa g

21

45

&lt;210&gt; 22

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

50

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide IC49

55

&lt;400&gt; 22

cggcaaaatc ccttataaat about





<213> Artificial sequence

<220>

<223> Description of the artificial sequence:  
oligonucleotide V198Gfor

<400> 27

ccgaatatca tgggggaaaa tggc

24

<210> 28

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:  
oligonucleotide V198Grev

<400> 28

gccattttcc cccatgatat tcgg

24

<210> 29

<211> 21

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:  
oligonucleotide D227Afor

<400> 29

ctaccctgctc tattgctgaa g

21

<210> 30

<211> 21

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:  
oligonucleotide D227Arev

<400> 30

cttcagcaat agcacgggta g

21

<210> 31

<211> 21

<212> DNA

<213> Artificial sequence

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide D227Vfor

5

&lt;400&gt; 31

ctaccctgtg tattgctgaa g

21

10

&lt;210&gt; 32

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

15

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide D227Vrev

20

&lt;400&gt; 32

cttcagcaat aacacgggta g

21

25

&lt;210&gt; 33

&lt;211&gt; 24

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

30

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide D261Gfor

&lt;400&gt; 33

35

gccttcttgg cgagttcttc tgag

24

40

&lt;210&gt; 34

&lt;211&gt; 24

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

45

&lt;220&gt;

<223> Description of the artificial sequence:  
oligonucleotide D261Grev

&lt;400&gt; 34

50

ctcagaagaa ctcgccaaga aggc

24

55

&lt;210&gt; 35

&lt;211&gt; 24

&lt;212&gt; DNA

&lt;213&gt; Artificial sequence

<220>

<223> Description of the artificial sequence:  
oligonucleotide D261Nfor

5 <400> 35  
gccttccttaa cgagttcttc tgag  
24

10 <210> 36  
<211> 24  
<212> DNA  
<213> Artificial sequence

15 <220>  
<223> Description of the artificial sequence:  
oligonucleotide D261Nrev

<400> 36  
20 ctcagaagaa ctcgttaaga aggc  
24

<210> 37  
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&lt;310&gt; PCT/EP/96/04631

&lt;311&gt; 1996-10-24

&lt;312&gt; 1997-05-01

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